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NANYANG
TECHNOLOGICAL
UNIVERSITY

**RAPID MONITORING OF GROSS TOXICITY IN
WATER AND WASTEWATER SYSTEMS THROUGH
MICROBIAL METABOLISM**

RAPHAEL ORTIZ

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2017

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RAPHAEL ORTIZ

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

13.8.2017

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Date

R. Ortiz

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Raphael Ortiz

Abstract

Biological treatment of wastewater represents a sustainable alternative to physicochemical processes. Efficiency is however hindered by the low tolerance of toxic loads. Herein, combining segmented-flow microfluidic and a cell viability assay for real-time gross toxicity detection and quantification was explored. The reduction of resazurin to fluorescent resorufin by bacteria was used to assess the toxicity of model toxicants pentachlorophenol (PCP) and five heavy metals Cu^{2+} , Hg^{2+} , Zn^{2+} , Ni^{2+} , Co^{2+} . A microfluidic chip was developed for use as a continuous microreactor, wherein 100 nL aqueous droplets are dispersed in a continuous oil phase. This platform can continuously generate sample droplets with sequential addition of bacteria and resazurin, allows determination of initial rates of resazurin reduction and dose dependent toxicity assessment with an overall response time of less than 5 minutes.

The assay was performed under alternative conditions from traditional laboratory procedures to reduce the response time, including short toxicant exposure (< 30 s), stationary phase bacteria at high density (3×10^7 cell mL^{-1}) and continuous mixing in moving droplets. A linear relationship was confirmed between cell density from 3.7×10^6 to 9.2×10^8 cell mL^{-1} and the resazurin reduction rate. Under optimized conditions, results were obtained within 3 minutes with better sensitivity than analogous microplate experiments. For instance, the apparent EC_{50} of PCP to *Enterococcus faecalis* was 6.3 mg L^{-1} in droplet format compared to 77 mg L^{-1} on microplate. The resazurin reduction rate increased with mixing in moving droplet up to a velocity of 4 mm s^{-1} . Under these conditions, Michaelis-Menten behavior was observed with respect to the starting resazurin concentration and its reduction was attributed to the presence of two or more different enzymes with apparent Michaelis constants of $\sim 1 \text{ }\mu\text{M}$ and greater than $140 \text{ }\mu\text{M}$ respectively. The ratio between enzymes was dependent on bacterial growth phase and they were selectively inhibited by Hg^{2+} and Cu^{2+} . Therefore, the apparent toxicity was highly dependent on the resazurin

concentration as well as the growth phase and 10 μ M resazurin was found optimal for *Enterococcus faecalis* in stationary phase.

The assay in droplet format was enabled by a novel surface modification technique relying on the unique stability of silica sols at their isoelectric point to adsorb a dense silica nanoparticle layer on PMMA. The silica layer acted as primer for surface silanization with alkyl trichlorosilanes that rendered the surface hydrophobic eliminating the need for surfactants and their inherent complications. Hexadecane supported continuous operation >72 h while fluorocarbons limited operation to 3-4 h because of fouling resulting in droplet pinning. The difference was attributed to repulsive Van der Waals interactions only with hexadecane as suggested by theoretical analysis of Hamaker constants. Effect of partitioning of hydrophobic molecules between aqueous droplets and the continuous oil phase was predicted by Hansen solubility parameters and studied with PCP. Perfluorocarbons were demonstrated to minimize the extraction of PCP ($\text{LogD}_{\text{oil/water}} = -1.47$) and hence underestimation of the toxicity compared to hydrofluoroethers ($\text{LogD}_{\text{oil/water}} = -0.66$) and hexadecane ($\text{LogD}_{\text{oil/water}} = +0.45$). Finally, suggestions are made to extend long-term performance with fluorinated continuous phases on the basis of Hamaker constant.

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- Figure 7.1** Erratic flow of aqueous droplets in fluorinated oil in channels ($700 \text{ }\mu\text{m}$ wide) treated with FOTS. (A) Droplets shape is asymmetrical and move in stepwise fashion, indicating inhomogeneous surface wetting. (B) Partial pinning in some area slow down the droplets, allowing the continuous phase to flow around them, thus reducing the spacing. (C) Injection of water into resorufin droplet. Pinning at the injection junction causes droplet to break further down the channel, causing cross-contamination with subsequent sample and irreproducible injection volumes.
- Figure 7.2** Representative AFM images of PMMA slides coated with one cycle of SiNPs, (A) without hydrophobic modification, (B) modified with DTS from solution, (C) modified with FOTS from solution ($>100 \text{ nm}$ objects were found on all scanned areas), (D) modified with FOTS by vacuum deposition.

- Figure 7.3** Visualizing FDTS surface modification in real time. FC-770 droplets in hexadecane are generated on silica coated PMMA. Thirty minutes after adding FDTS to FC-770 phase, wetting is completely reversed and hexadecane droplets in FC-770 are formed.
- Figure 7.4** Wetting transition of aqueous droplets in fluorocarbon oil on DTS coated channels. (A) Normal droplets generation under dynamic conditions. (B) After pausing the flow, part of the droplets remains pinned. (C-E) Schematic representation, (A) Under dynamic conditions, viscous entrainment maintains a lubrication layer between the droplet and the wall, (B) When the flow is stopped, the layer vanishes and droplets rapidly transition to the Wenzel wetting regime, (C) Upon resuming the flow, pinned droplets are broken or deform until the channel is only partially obstructed.
- Figure 7.5** (A) Scheme to increase PMMA surface roughness by solvent swelling. (B-C) Low and high magnification AFM images of PMMA exposed to 60% acetone for 60 s. (D-E) corresponding images after subsequent SiNPs coating.
- Figure 7.6** (A) PMMA roughness as function of acetone treatment time. (B) Droplets in fluorocarbon oil in microfluidic chip treated for 60 s prior to hydrophobic modification. (C-D) AFM images of PMMA exposed to 60% acetone for 30 and 120 s.
- Figure 7.7** (A) Estimated Non-retarded Hamaker constants for water in oil on different substrates. (B) Expected wetting regimes under static conditions.
- Figure 7.8** Absorbance calibration curves for PCP in (A) PBS and (B) hexadecane.

Figure 7.9 PCP distribution coefficients between PBS and various oils.

Figure 7.10 Effect of continuous phase on the dose response of *E. faecalis* to PCP assessed by resazurin reduction in microfluidic format.

Figure 7.11 Resazurin reduction by *E. faecalis* spiked with different PCP concentrations, measured in microplate by Dr. Chen Jianlin. Resazurin concentration was 100 μ M versus 10 μ M used in microfluidic experiments. Inset shows the reduction rate in the first 10 minutes.

Figure 8.1 Resazurin reduction test with anaerobic sludge.

Figure 8.2 Water, hexadecane, fluorocarbon triple phase systems in channels modified with silica and, (A) DTS (B) FDTS.

Abbreviations

4PL	4 Parameter Logistic regression
AFM	Atomic Force Microscopy
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion
CA	Contact Angle
Ca	Capillary number
COD	Chemical Oxygen Demand
DI	Deionized
DTA	Direct Toxicity Assessment
DTS	Dodecyltrichlorosilane
EC50	Half Effective Concentration
FC	Fluorocarbon
FDAc	Fluorescein Diacetate
FDTs	1H,1H,2H,2H-Perfluorodecyltrichlorosilane
FEP	Fluorinated Ethylene Propylene
FESEM	Field Emission Scanning Electron Microscopy
fps	Frame per Second
GF-AFC	Glycyl-phenylalanyl-amino-fluorocoumerin
HFE	Hydrofluoroether
HMDSO	Hexamethyldisiloxane
HRT	Hydraulic Retention Time
IC50	Half Inhibitory Concentration
ID	Internal Diameter
LbL	Layer by Layer
LED	Light Emitting Diode
LOD	Limit of Detection
MFC	Microbial Fuel Cell
MIC	Minimum Inhibitory Concentration

MM	Michaelis-Menten
MoG	Mixture of Gaussian
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NIC	Non-Inhibitory Concentration
PBS	Phosphate Buffered Saline
PCP	Pentachlorophenol
PDMS	Polydimethylsiloxane
PE	Polyethylene
PET	Polyethylene Terephthalate
pKa	acid dissociation constant
PMMA	Poly(methyl methacrylate)
PVC	Polyvinyl Chloride
SAM	Self-Assembled monolayer
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SiNP	Silica Nanoparticle
U.S. EPA	U.S. Environmental Protection Agency
UV	Ultraviolet
VFA	Volatile Fatty Acids
WET	Whole Effluent Toxicity
YPD	Yeast extract Peptone Dextrose

Chapter 1

Introduction

Real-time toxicity assessment of wastewater is a major obstacle towards more sustainable biological treatments. Current techniques are limited to indirect online monitoring of physical parameters or delayed offline toxicity analysis. As a result, biological systems for wastewater treatment are operated at suboptimal conditions and with large safety factors to avoid toxic inhibition. The size and energy consumption of treatment plants could be substantially reduced by high rate processes if inhibitory wastes were detected within minutes, preferably quantitatively. Herein, combining segmented-flow microfluidic and a cell viability assay for real-time gross toxicity detection and quantification is proposed. This chapter presents the hypothesis and project objectives and briefly overviews each chapter as well as scientific contributions.

1.1 Background

Despite water covering over 70% of the earth surface, only 2.5% is fresh water, out of which about 1% is easily accessible. Because of growing needs and increasing population, it is estimated that by 2025, 1.8 billion people will face water scarcity [1]. Therefore, much effort is needed to recycle and prevent pollution of available resources.

Natural water resources including lakes, rivers or groundwater are interconnected to industrial and domestic usage through complex distribution and sewer networks involving storage, purification and recycling facilities which constitutes as many vulnerabilities to pollution. Protective measures primarily rely on online monitoring of physical parameters (pH, dissolved gas, temperature, turbidity, etc.) while toxicity assessment still heavily rely on manual sampling [2]. Real-time toxicity monitoring is currently limited by long response time and poor detection limits but would be useful in several locations for monitoring against bioterrorism, enforcing discharge regulations as well as protection of biological treatment plants. In particular, anaerobic digester used to treat wastewaters could benefit from early toxicity warning. More specifically, high rate anaerobic digesters that have emerged has sustainable alternative compared to conventional biological treatments [3] [4]. They hold great promises including lower energy consumption, smaller footprint, reduced excess sludge and biogas production; potentially equating to an overall positive energy balance. Widespread use has however been impeded by the relative greater sensitivity of anaerobic digestion to toxicants compared to activated sludge. If corrective measures are not taken, toxic shocks not only prevents degradation of the wastes, but can take weeks to recover from [5]. The window for action is especially narrow for high rate digesters and to be useful, toxicants should be detected in minutes.

While knowing the exact composition of the influent would be ideal, it is chemically impracticable and financially impossible to obtain in real time. The next best option, without being restrained to a specific target, is a gross toxicity assessment. Cell viability assessments are procedures commonly encountered in laboratory. As an example, the reduction of the blue, non-fluorescent compound resazurin to pink, fluorescent resorufin is

used to assess cell or bacterial viability as illustrated in **Figure 1.1**. Protocols reported for susceptibility testing are labour-intensive and typically require 1 to 4 hours of incubation. However, resazurin signals can be detected in as little as 10 minutes in severely contaminated milk suggesting faster detection is possible [6].

Figure 1.1 Resazurin viability indicator. Non-fluorescent resazurin is irreversibly reduced by living cells to fluorescent resorufin ($\lambda_{\text{max}} = 590 \text{ nm}$).

Furthermore, microfluidic technology has emerged as a method to automate sample preparation and detection. Recent advances in multiphase microfluidic allow for high-throughput scanning of multi-parameter systems. Typically, controlled aqueous droplets are formed in a continuous oil phase and act as micro-reservoirs to carry out various isolated reactions. Miniaturization leads to several advantages; reduced reagent consumption, multiplexing ability, and faster response time. We therefore aim to leverage these advantages to develop a real-time toxicity sensor.

1.2 Hypothesis

Hypothesis #1: *Measuring the kinetics of microbial reductases via droplet generating microfluidic chip allows real-time dose-dependent influent toxicity assessment.*

Sub-hypothesis #1: *Hydrophobic surface modification of the channel walls prevents wetting of the walls by the dispersed aqueous phase without surfactant additives.*

Sub-hypothesis #2: *Larger Hansen solubility distance between the continuous oil phase and toxicant reduces its partitioning from the dispersed aqueous phase and decreases its apparent half inhibitory concentration.*

Sub-hypothesis #3: *Toxicants have a unique response to multivariate (microorganism, growth stage, resazurin concentration) analysis of their dose-response relationship.*

1.3 Objectives and scope

The first objective was to develop a droplet based microfluidic platform allowing continuous and automated sample generation and fluorescence kinetics recording that enable the resazurin assay to be performed under conditions resulting in less than 5 minutes delay. The second objective was to investigate, under these new conditions, the effect of substrate, microorganism and inhibitor concentrations as well as continuous mixing in droplet and phase partitioning on the resazurin kinetics.

To achieve these objectives, the following work was carried out:

- PMMA Microfluidic chip fabrication, hardware and software setup development and initial testing using surfactant stabilized water droplets separated by hexadecane.
- Hydrophobic surface modification of enclosed microfluidic channels based on adsorption of silica nanoparticles.
- Logarithmic serial dilution in droplet microfluidic with integrated micromagnetic stirrer to generate dose-response curves.
- Resazurin reduction kinetics as function of flow rate, bacterial density and growth phase, initial resazurin concentration and inhibition mechanisms of Cu and Hg.
- Comparison of Cu, Hg, Co, Ni, Zn inhibition towards *E. faecalis* and *saccharomyces cerevisiae*.
- Adaption of surface modification to fluorocarbon as continuous phase
- Oil-water distribution coefficients of pentachlorophenol (PCP) in hexadecane, hydrofluoroether and perfluorinated liquids.
- Apparent PCP toxicity as function of the continuous phase and comparison with analogous microplate experiments.

1.4 Dissertation Overview

This thesis consists of the following 8 chapters:

Chapter 1 provides a rationale for the research and outlines the objectives and scope.

Chapter 2 provides the literature review that lead to the pre-stated hypothesis, including bioassays/biosensors and microfluidic applications relevant to water toxicity.

Chapter 3 discusses the strategies and rationale for materials and methods selection, including the choice of model toxicants and model organisms, the analysis of kinetics and inhibition curves, microfluidic design considerations and strategies for surface modification.

Chapter 4 introduces the microfluidic hardware and image processing software that was developed to monitor fluorescence kinetics in droplets.

Chapter 5 describes the permanent surface modification of PMMA channels to avoid surfactant stabilized droplets and discusses the trade-offs imposed by hexadecane as the continuous phase.

Chapter 6 addresses the refinement of the microfluidic platform to create logarithmic concentration gradients enabling complete dose response curves. A detailed analysis of the resazurin kinetics is presented as well as a comparison of heavy metals inhibition on bacteria and yeast.

Chapter 7 refines the surface modification for fluorinated continuous phase and extends the toxicity assessment to hydrophobic compounds.

Chapter 8 summarizes the results and reevaluates the hypotheses for further improvements and future research.

1.5 Findings and Outcomes/Originality

This research led to several novel outcomes by:

1. Demonstrating the adsorption of silica nanoparticles at their isoelectric point on plastic substrate to form a dense but conformal layer of sub-micron features that act as a primer for further derivatization.
2. Developing a microfluidic platform to perform a viability assay in just 3 minutes with better sensitivity than its microplate equivalent.
3. The resazurin reduction rate is dependent on flow-rate mediated mixing in droplet based microfluidics.
4. Attributing the resazurin reduction to multi-enzyme Michaelis-Menten kinetics and their selective inhibition.
5. Demonstrating the tradeoffs of hydrofluoroether in segmented-flow microfluidic due to phase partitioning and quantitatively evaluating its impact on bioassay in droplet format.

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Chapter 2

Literature Review

This chapter surveys the existing wastewater monitoring techniques, microorganisms of interest and relevant toxicants with emphasis on early warning of anaerobic digestion upset. Whole-cell based biosensors are attractive for gross-toxicity monitoring but rely on immobilized organisms making recovery after toxic events difficult or impossible. Typical response time range from 30 min to several hours, thus limiting their use as early warning. Laboratory-based cell viability assays on the other hand are inherently robust due to multiple replicate and control samples but while detectable signal can be obtained in minutes, the overall protocols are long and labor intensive. Microfluidic counterparts to bioassays potentially serviceable as sensors are discussed. Droplet based microfluidic is introduced and concentration gradient generation, related surface modification techniques and the role of surfactants as well as existing limitations are examined. Surfactants used to control interfacial properties in two-phase microfluidics prevent droplet coalescence, increase solute transfer and affects microbial metabolism and thus would be best avoided whenever possible. Moreover, fluorinated continuous phases have been recognized as a better alternative to hydrocarbons to prevent leakage from droplets but difference among them, in particular between hydrofluoroethers and perfluorocarbons has not been addressed quantitatively.

2.1 Central Hypothesis

Measuring the kinetics of microbial reductases via droplet generating microfluidic chip allows real-time dose-dependent influent toxicity assessment.

2.1.1 Online Biosensors for Wastewater Monitoring

Supposition 1: *“Conventional” online biosensors lack stability and precision.*

A range of advanced techniques coupled to multivariate analysis have been developed over the years to monitor reactor operating conditions [1-2]. It has however limited utility in preventing toxic inhibition as the effect of toxic loads is reflected with a substantial lag. Thus, it is often too late to take proactive measures when abnormalities are detected. We therefore focus the following review on existing solutions that can provide an early warning by monitoring influent before it reaches the reactor.

In an effort to provide test conditions similar to a full-scale reactor, Rozzi et al. have proposed to use a laboratory scale (5L) anaerobic reactor upstream of the main reactor [3-5]. Unlike normal operating conditions, the reactor was periodically spiked with acetic acid to ensure that methanogens operate under kinetically saturated conditions. This is an important consideration as it emphasizes the effect of toxic compounds. NaCl and chloroform toxic loads were related to the volume of methane produced with good correlation, albeit a slow response time of ca. 2 hours. However, no follow-up reports exist in the academic literature or commercial publications. Complexity of operating two simultaneous bioreactors is a major drawback.

In a similar manner, Jiang et al. employed a small reactor (~1L) with anaerobic granules as a ‘Canary in a coal mine’ sensor for a larger activated sludge reactor [6]. Change of redox potential was monitored in the anaerobic reactor and showed a positive correlation with chemical oxygen demand (COD) and ammonia nitrogen removal of activated sludge upon addition of various concentrations of copper (II) and phenol. The response time was

1-4 h, within the advance time needed of their aerobic reactor operated at hydraulic retention time of 12 h (vs 6 h for the sensor). It should be noted that it took 40 to 100 hours to recover the original redox potential after shock loading. Significant baseline drift was also observed.

Microbial fuel cells (MFC) initially developed as renewable source of energy can also serve as biosensor as recently reviewed [7]. A MFC has been shown to have a near linear relationship between the current produced and COD of the feed and a response time of 40 min [8]. Reduced response time can be attributed to the small size of analytical MFCs. Dávila et al. demonstrated a MFC with an internal volume of only 144 μ L that allowed a near instant response [9]. However, the MFC was not able to recover when toxicant was removed. That is one of the main drawbacks of MFCs; the timescale to grow an electro-active biofilm is a matter of days and recovery after toxic load is not always possible. It should also be noted that electro-active biofilms, can present a high resistance to a wide range of toxicants including antibiotics and heavy metals [10] and might therefore not be representative of the biomass response in anaerobic digester.

Apart from the above applications that are especially relevant to anaerobic processes, a plethora of whole-cell based sensors for water monitoring have been developed over the years. Su et al. recently reviewed microbial biosensors [11]. For instance, the Microtox assay relies on *vibrio fischeri* bacteria, a naturally occurring example of bioluminescence. However relevancy of the test has been criticized due its marine origin [12]. To overcome this issue, other organisms (predominantly *E.coli*) have been genetically modified to produce bioluminescence or fluorescent product such as green fluorescent protein [13]. Faster response are reported for whole-cell bioluminescence sensors but still range within 0.5-2 h [14]. More importantly, the luciferase reaction requires the presence of oxygen and is therefore not compatible with anaerobic organisms.

While some of the biosensors have short enough response time; they have some major drawbacks for the targeted application. Baseline drift and repeatability are generally an issue for online monitoring, but the major disadvantage is the long starting and recovery time after toxic events (if possible at all). These approaches seem suitable for situations

where toxicity is not expected under normal conditions such as monitoring against bioterrorism or illegal waste disposal. However, in small scale, high rate anaerobic reactors, one can expect to encounter significant toxic loads repeatedly and in unpredictable manner. A bioassay approach where potentially toxic samples are periodically generated and assayed would provide a more robust early warning system for microbial bioreactors.

2.1.2 Cell Viability Assays

Supposition 2: *Resazurin assay allows for microbial metabolism assessment within minutes including the one of anaerobic bacteria.*

Resazurin, is a blue, non-fluorescent compound that is irreversibly reduced inside cells by reductases, to resorufin, a pink and highly fluorescent compound (**Figure 1.1**) [15]. It was first reported by Pesch and Simmert in 1929 as an indicator for contamination of milk by bacteria or yeast [16]. Since then, it has been extensively used as a quantitative method for cell counting and viability assessment [17]. It should be pointed out that resorufin can be further reduced, reversibly, to dihydroresorufin which is transparent and non-fluorescent. This can lead to erroneous interpretation when fluorescence is measured at a single time point [15]. Nonetheless it can be easily identified by monitoring the fluorescence kinetics, at least during establishment of a new protocol. Additionally, although resazurin has been reported to have toxic effect on cell culture over several days, it is generally regarded as nontoxic on shorter timescale [18]. Finally, while dependent on microorganisms and density, fluorescence signals are generally detectable within minutes in healthy organisms which makes it a good candidate for rapid detection [19].

Figure 2.1 Resazurin viability indicator. Non-fluorescent resazurin is irreversibly reduced by living cells to fluorescent resorufin ($\lambda_{\text{max}} = 590 \text{ nm}$) and subsequently reversibly, in the presence of oxygen to non-fluorescent dihydroresorufin.

In relation to the present context, resazurin has been used for biomass activity test of activated sludge [19-20] as well as to measure toxicity of 2,4 dichlorophenol on pure bacterial culture [21] and activated sludge [22]. To a lesser extent, it has also been used for the assessment of anaerobic bacteria. It is worth noting that anaerobic culture mediums might contain reducing agents such as L-ascorbic acid that can reduce resazurin in the absence of cell. Consequently, Natto et al. based their assay for *trichomonas vaginalis* (aerotolerant) on the second reduction from resorufin to dihydroresorufin [23]. In contradiction, Karakashev et al. reported that facultative and aerotolerant anaerobes can only convert resazurin to resorufin while no conversion is observed with strict anaerobes [24]. However more investigation is needed as they only used one strain from each class and their experiments were solely based on qualitative visual observations.

Resazurin is widely used, well validated and economically advantageous. Nevertheless, other reagents are available for viability assay and compared in **Table 3-1** [25-27]. Tetrazolium based substrates are also popular but rely on absorption measurement which is usually less sensitive than fluorescence. Furthermore, absorption methods are ill-suited for microfluidic applications where the absorption paths are typically hundred times shorter than in cuvette [28]. ATP bioluminescence is also widely used as it is fast and sensitive, but no kinetic study is possible as it is an endpoint assay. It is also sensitive to temperature variations. Moreover, it requires the presence of oxygen which is problematic with anaerobic culture and inside a microfluidic channel. More recently, assay based on glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC) have been developed. Fluorescent AFC is released when GF-AFC broken down by intracellular proteases. It is also part of a live/dead

cell assay [29]. Use with bacteria has however not been reported and it is 1000 times more expensive than resazurin. Finally, fluorescein diacetate (FDAC) and many of its derivatives can be used for viability assessment based on esterase activity. While its derivatives are generally designed to increase retention inside the cell after hydrolysis, making them more suitable for flow cytometry and fluorescence imaging, FDAC would be an economical alternative in the present context. It is however poorly soluble in water (logP of 4.2 [30]), subject to self-quenching [31] and has limited photostability [32]. In conclusion, the resazurin assay is well proven, fast and economical, hence seems to be the ideal reagent for a microfluidic toxicity assay because it allows rapid detection in continuous fashion.

2.1.3 Droplet Microfluidic

Supposition 3: *Two-phase microfluidic allows for high throughput, automated assay with reduced reagents consumption.*

Bioassays are usually associated with costly and labour intensive procedures. However, some of these procedures can be automated, thus blurring the line between biosensors and bioassay [33-34]. For instance, the commercial Toximeter NitriTox from LAR is an example of automated respirometric toxicity assay applied to wastewater. Furthermore, reagent consumption can be greatly reduced by scaling down the assay to microfluidic size where droplet volume is on the order of microliters to picolitres making continuous operations with high sampling frequency more viable.

Several bacterial viability assays based on microfluidic chips have been proposed. For instance, Elavarasan et al. reported a resazurin antibiotics efficacy assay with pre-dried reagents on a Poly(methyl methacrylate) (PMMA) chip [35]. Perfusion of cell cultures with 1-dimensional gradient have been reported with resazurin [36] and calcein [37] as indicator as well as 2-dimensional gradients [38-39]. These approaches work well under static conditions, however under dynamic conditions sample preparation is difficult due to Taylor-Aris dispersion and slow mixing [40].

Microfluidic droplet reactors have multiple prospects from analytical to synthesis applications [41]. Various techniques for droplet generation and manipulation have been developed [42]. Droplets can be generated continuously at rate up to a few kHz due oil/water interfacial instabilities. In this way, all droplets have the same content and can be useful for statistical analysis such as single cell encapsulation [43]. Droplets with varied content can also be generated by creating temporal concentration gradients as elaborated in the next section. Majority of droplet-based bioassays focus on high throughput screening rather than continuous operation and fast response time [44]. Nevertheless, we highlight two relevant works that relate to our technology. Churski et al. reported a platform for antibiotic screening using *E. coli* and resazurin [45]. Their procedure mimics the standard resazurin protocol. After generation, droplets were loaded in a section of tubing and incubated for 4 hours before reinjection and a single fluorescence reading was done. Cho et al. studied photosensitizer activity on *E. coli* [46]. After incubation in an external chip, they re-injected the droplets to perform a live/dead cell assay on chip. In addition, segmented flow has also been used to reduce dispersion in online micro-dialysis probes for monitoring glucose [47] and hydrogen sulphide [48]. Micro-dialysis sample droplets have also been collected and stored for offline capillary electrophoresis of neuroactive amino acids [49].

In the present application, a droplets-based approach seems therefore adequate to repeatedly generate a sequence of samples, i.e. positive/negative controls and a few test conditions at regular intervals while minimizing reagent consumption and avoiding temporal dispersion.

2.1.4 Microfluidic Concentration Gradient Generator

Supposition 4: *Concentration gradients in microfluidic offer unmatched opportunity to quantify toxicity.*

Given the number of emerging pollutants but also unknown additive or synergistic effects of recognized toxicants, non-specific detection of gross toxicity is the only viable option for early warning system. To decide on the corrective measures to be taken (waste dilution,

pre-treatment, etc.), the ensuing question is however, how toxic is it? Unlike sensors or assays for specific compounds, no calibration can be done in advance. Instead, dilution series of the sample is performed following protocols referred to as direct toxicity assessment (DTA) [50] or, specifically for water samples, whole effluent toxicity (WET) [51]. Droplet microfluidic is particularly suited for this task and several methods have been developed to generate concentration gradients in droplet trains.

Figure 2.2 Methods to generate concentration gradients in segmented-flow microfluidic, (A) relative aqueous flow rates variation, (B) Batch mode injection, (C) Spatial gradient split in parallel droplet stream, (D) Segmentation of Taylor-Aris dispersion profiles, (E) Droplet trap serial dilution, (F) Serial dilution from external stirred well.

Droplets content can be adjusted, not only to create positive and negative control droplets but also intermediate concentrations. The most straightforward technique is to adjust the relative flow rates between reagents [52] (**Figure 2.2A**) or controlling volumes injected

from pressurized reservoirs through valve opening time [45, 53] (**Figure 2.2B**). With the later, droplets are truly independent, albeit generated at lower rate ($\sim$ Hz), and complex combinatorial pattern can be achieved. With both however, the practical concentration range is limited to about 2 orders of magnitude because flow rates or valve opening time must vary within the same dynamic range. Hydrodynamic resistor networks developed for single phase microfluidic have also been combined with parallel droplets generation to create linear [54] and logarithmic [55] concentration profiles (**Figure 2.2C**). Due to the limited space available in one chip, this parallel approach limits the number of concentrations (< 10) and on-chip incubation time but generate a large number of replicates. While it useful for statistical analysis of single enzyme or single bacteria, it is unnecessary when only the average response is considered. In contrast, high resolution concentration gradients can be obtained by segmentation of a Taylor-Aris dispersion profile into droplets [56-57] (**Figure 2.2D**). Such concentration profile depends on the diffusion coefficient of the solute which can be compensated for if known but it is a major drawback for mixture or unknown samples. Finally, serial dilution by merge and split operations of droplets can generate gradients spanning up to 4 orders of magnitude in continuous flow [58] or static array [59] (**Figure 2.2E**). The throughput is limited by the large fraction of oil needed between droplets to completely stir the content between each operation. This limitation can be alleviated at the cost of an extra active stirring element which has recently been demonstrated from an open microwell under magnetic stirring [60] (**Figure 2.2F**). With regards to toxicity testing, gradient over a wide range are beneficial to cover the entire dose response curve. Conversely, high resolution allows more accurate representation of variations happening in narrow concentration range. High throughput of microfluidic technologies offer a unique opportunity to do both simultaneously. As toxicity warning for bioreactor, it will be useful to determine how much dilution is needed to treat the waste and determine safety margins.

2.2 Sub-hypothesis 1

Hydrophobic surface modification of the channel walls prevents wetting of the walls by the dispersed aqueous phase without surfactant additives.

2.2.1 Surfactants in Microfluidic

Supposition 1: *Surfactants are often used to control interfacial properties in two-phase microfluidics. However, surfactants, prevent droplet merging, increase solute transfer and affects microbial metabolism.*

Surfactants are generally added to the continuous phase to prevent droplet coalescence, but also control wetting of the channel walls by the dispersed phase [61]. For instance, by using adequate surfactant, it is possible to prepare W/O or O/W emulsion in PMMA chip [62]. It however introduces several shortcomings including increased level of background fluorescence [63] and varied microbial metabolism [64]. Moreover, as surfactant concentration increases, merging droplet becomes impossible without active measure such as electro-coalescence which complicates the fabrication process and experimental setup [65]. Droplet stabilization may be unnecessary if droplet contact and post-microfluidic operations are minimized.

Control of surface wetting has also been used, mostly on PDMS [66] and glass [67] and to a lesser extent on plastic [68-71] chips. Surprisingly, surfactants are almost invariably added in conjunction to the reported surface modifications. A notable exception is the work by Jankowski et al. where they reported the covalent surface attachment of dodecylamine to produce stable water and alginate droplets in hexadecane [71]. The improved wetting properties were due to hydrophobicity, but also to an increase in surface roughness as elaborated in the next section. While their approach is specific to polycarbonate and adapted to hydrocarbon oils, it strongly suggests that surfactants can be avoided when droplets are not in direct contact with each other.

2.2.2 Surface Wetting of Water Drop in Oil

Supposition 2: *In oil, water repellency can be thermodynamically stable, even on flat surface.*

Nature inspired superhydrophobic surfaces generally rely on air pockets trapped within the rough surface texture [72]. This Cassie wetting regime illustrated in **Figure 2.3** is often metastable and eventually water irreversibly penetrates within because of pressure variations or gas diffusion. This transition to a Wenzel wetting regime is characterized by the drops sticking to the surface [73]. More recently, lubricant impregnated surfaces, where pockets are filled with oil instead of air, have been introduced as a more robust alternative [74-76] and excellent anti-biofouling properties have been demonstrated compared to traditional superhydrophobic and PEG-functionalized surfaces [77]. Furthermore, under dynamic flow conditions within microchannels, an oil lubrication film is formed and prevents droplets from wetting the wall [78]. As the velocity is reduced the lubrication film becomes thinner allowing intermolecular forces between the droplet and microfluidic wall to dominate [79].

Figure 2.3 Cassie and Wenzel wetting regimes for a water droplet on a rough substrate.

Beside incompressibility of the impregnating oil, there is another fundamental difference than can account for the excellent stability of lubricated surfaces. Van-der-Waals interactions between a droplet and a solid hydrophobic surface is dependent on the intervening medium and can be quantified by the Hamaker constant. When the dielectric function of the intervening medium is intermediate between the two interacting bodies, the Hamaker constant becomes negative thus indicating the presence of repulsive forces. This condition can never be satisfied for interactions across air but can be met for certain oil-water systems [80]. While most studies consider pure liquids, in real application the presence of surface active molecules might have a significant effect. Geerken et al. [81]

studied wetting between water and hydrocarbons or fluorocarbons monolayers across hexadecane. In the presence of emulsifier, the drops started to spread on fluorocarbons coating but not on hydrocarbons ones. They attributed this phenomenon to unfolding and adsorption with hydrophilic groups facing outwards of proteins present in the emulsifier, triggered by the lower surface energy ($< 15 \text{ mN/m}$) of fluorinated coating. Altogether it suggests that with the right combination of surface coating and continuous phase, long-term droplet generation is possible without surfactant but, too low surface energy could be detrimental when bacteria culture containing proteins is introduced.

2.3 Sub-hypothesis 2

Larger Hansen solubility distance between the continuous oil phase and toxicant reduces its partitioning from the dispersed aqueous phase and decreases its apparent half inhibitory concentration.

2.3.1 Molecular Transport in Two-phase Microfluidic

Supposition 1: *Droplets are not perfectly isolated microreactors.*

Indeed, transfer of water to neighbouring droplets due to osmotic mismatch results in size variation and has been used to monitor bacterial activity [82]. Transfer between droplets is possible through reverse micelles or bi-layer formation for contacting droplets [83-84]. Therefore, reduction of surfactant concentration as discussed above would be advantageous.

Droplet content can also simply leach to the oil phase due to unfavourable partition coefficient. In fact, it has been used for on-chip liquid-liquid extraction [85-86]. When this effect is undesirable, fluorinated oil phases are generally used because of their inability to dissolve non-fluorinated molecules [87]. It also provides better biocompatibility over hydrocarbon and silicone oils [61]. Selection of fluorinated solvents is however seldom detailed and there is a lack of quantitative data supporting these choices. For instance, the more environmentally friendly hydrofluoroether (HFE) oils such as 2-trifluoromethyl-3-ethoxydodecafluorohexane (HFE-7500) is very popular [88-90], despite having been

shown to exchange resorufin between droplets 5 times faster than fully perfluorinated oil Perfluorotri-n-butylamine (FC-40) [91]. Furthermore, solubility of fluorocarbon can change drastically with temperature and it should be considered when incubated at biological temperature [92]. Many partition coefficients have been reported in the literature, but the focus is on fluorinated molecules and solvent systems involved in fluorous chemistry [93].

In essence, fluorinated solvents are the best option, but quantitative data are needed to choose the right one. In particular, non-fluorinated hydrophobic molecules such as chlorophenols, halogenated aliphatic compounds and long chain fatty acids are relevant to wastewater applications [94].

2.4 Sub-hypothesis 3

Toxicants have a unique response to multivariate (microorganism, growth stage, resazurin concentration) analysis of their dose-response relationship.

2.4.1 Comparison of Inhibitory Concentration Range Between Microorganisms

Supposition 1: *Toxicity patterns vary greatly among microorganisms.*

Microorganisms are considered non-specific reporter, nonetheless inhibition by different toxicant varies greatly with the selected species as illustrated in **Table 2-1**. Using the resazurin assay, Zare et al. have measured the half effective concentration (EC50) of Cd, Hg and Pb on four major bacteria present in aerobic digestion [95]. EC50 varied up to 40-fold between species but in all cases the toxicity ranking remained Cd > Hg > Pb. In comparison, Cd has been reported to be 10 times less toxic to *Vibrio fischeri* (Microtox bioluminescence assay) while Hg and Pb were a 100 times more toxic and ranked as Hg > Pb > Cd [96]. Kurvet et al. also compared the toxic effect of 4 heavy metals and 3 organic chemicals between *Vibrio fischeri* and luminescent *E. coli* and noted that, despite slight differences, there was a good correlation ($R^2 = 0.7$) between the two and argued that comparable response was due the same inhibition mechanism leading to decrease in

bioluminescence [97]. Codina et al. studied the toxicity of 6 heavy metals (Cd, Cu, Zn, Cr, Ni, Hg) to *Saccharomyces cerevisiae* (Yeast), *Pseudomonas fluorescens* and *Vibrio fischeri* with in total 6 different assay methods [98]. Overall yeast was the least sensitive and toxicity ranking was similar among the three with the notable exception of chromium that was the most toxic only to yeast.

Table 2-1 Comparison of reported EC50 of selected heavy metals on various organisms

Bacteria	Assay	Ref.	EC50 (mg/L)			
			Hg	Pb	Cd	Cr
<i>Pseudomonas</i>	resazurin	[95]	2.35	19	0.007	-
<i>Aeromonas</i>	resazurin	[95]	8.4	10	0.05	-
<i>Enterobacter</i>	resazurin	[95]	9.5	32	0.015	-
<i>Bacillus</i>	resazurin	[95]	13	200	0.3	-
<i>Vibrio fischeri</i>	bioluminescence	[96]	0.1	0.46	0.51	-
<i>Vibrio fischeri</i>	bioluminescence	[97]	0.23	-	31	-
<i>E. coli</i>	Eng. bioluminescence	[97]	0.049	-	3.49	-
<i>Vibrio fischeri</i>	bioluminescence	[98]	0.2	-	35	340
<i>Pseudomonas fluorescens</i>	INT reductase activity	[98]	9	-	53	82
<i>Saccharomyces cerevisiae</i>	INT reductase activity	[98]	101	-	600	12.3
<i>Saccharomyces cerevisiae</i>	Growth (optical density)	[99]	15	-	1.1	-

There are substantial variations of reported EC50 for the same toxicant and bacteria between studies. This is particularly surprising for *Vibrio fischeri* (Microtox) which is used as reference in several studies. Some of the authors pointed out that EC50 could vary up to 1000 folds depending on the medium used with particularly large variability observed with zinc, probably because of different speciation. Absolute EC50 also varies with the assay used but the toxicity ranking for a given species is consistent between studies. Overall, meeting lower detection limits set by environmental agencies is challenging for gross toxicity assessment [14], but for applications like biological wastewater treatment, if not carefully selected, reporting organisms could very well be too conservative. In addition, the fastest of these assays require 15 to 30 min incubation which means that beside

automating the process with microfluidic techniques, the assay conditions will have to be changed to shorten the response time.

2.4.2 Susceptibility of Anaerobic Digestion to Toxicants

Supposition 2: *Methanogens are often regarded as the most sensitive to toxicants in anaerobic digestion.*

We briefly introduce the mechanisms of anaerobic digestion and the effect of toxic loads. For a comprehensive overview of anaerobic process, we refer the reader to the review by Appels et al. [100]. While focused on the treatment of waste-activated sludge, i.e. waste with high solid content, presented concepts cover anaerobic digestion in general.

Anaerobic digestion is a complex process where a wide variety of organisms work in concert to convert organic substances into methane and carbon dioxide. This process can be divided into four main stages as shown in **Figure 2.4**. First, large particles are broken down into soluble compounds by hydrolysis. Second, during acidogenesis, intermediary metabolites are broken down into volatile fatty acids (VFA), acetic acid as well as carbon dioxide and hydrogen. Remaining VFAs are further converted into acetic acid, hydrogen and carbon dioxide during acetogenesis. Finally, methanogens, classified as archaea, produce methane from acetate, hydrogen and carbon dioxide [101].

Figure 2.4 Main stages of anaerobic digestion.

Different steps can be rate limiting depending on the nature of the feed and operating conditions. For instance, hydrolysis of insoluble particles is often regarded as the rate limiting step for wastes with high solid content [100]. On the contrary, methanogenesis is regarded as the limiting factor for digestion of soluble wastes. In particular, the slow growth of acetoclastic methanogens (producing methane and carbon dioxide from acetic acid) can result in accumulation of acetic acid during transient conditions [102]. In addition to complex coupled reactions, methanogen population is often considered the most susceptible to toxicants [103].

Leitão et al. reviewed the effect of operational and environmental variations on anaerobic digestion systems [104]. They discussed the effects of temperature and pH variations, change of loading rate, as well as the nature of carbon source. Toxicant inhibition, including ammonia, sulfides, heavy metals and some organics was reviewed by Chen et al.

[105] and more recently by our group [94] with emphasis on inhibition mechanisms and inclusion of nanomaterial as emerging contaminant.

2.5 Summary

In conclusion, toxicity monitoring can prevent biological reactor upset if available in real-time, i.e. less than 5 minutes. The resazurin viability assay could be used to monitor water toxicity granted that it is performed automatically. Segmented-flow microfluidic has the potential to transform this traditional laboratory procedure into a continuous monitoring device and the assay conditions will also have to be adapted to produce detectable signals more rapidly.

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Chapter 3

Experimental Methodology

This chapter introduces the overall approach taken to develop a rapid toxicant detection method in microfluidic format. The first part covers the rationale for selection of the cell viability assay, reporting organisms and model toxicants as well as standard methods used for validation. Analysis of kinetics and dose response curves are also presented. In the second part, selection of microfluidic droplet format is justified and the choice of materials, fabrication method and surface modification approach are discussed. At last methods for surface characterization are examined including rapid assessment by dynamic contact angle measurements and detailed surface morphology analysis by atomic force microscopy and scanning electron microscopy.

3.1 Overview of the Approach

The objectives were developing a new microfluidic platform to perform the resazurin assay automatically and in continuous fashion as well as optimize and validate assay conditions suitable for continuous toxicity monitoring in less than 5 minutes. The following features were targeted:

1. Droplet format. Each aqueous droplet in a continuous oil phase constitutes a sample.
2. Sequential reagents injection to ensure microorganisms are exposed to toxicants before resazurin and prevent cross contamination between reagents.
3. Time course fluorescence intensity collection to determine resazurin conversion rates.
4. Surface modification alleviating the use of surfactant.
5. Adjustable concentration of toxicant for dose response analysis.
6. Fluorinated continuous phase to prevent extraction of hydrophobic toxicants.

Developing the microfluidic droplet version of a viability assay and hypothesis testing for this project involved a lot of intertwined components that were approached as follow. In the first stage described in **Chapter 4**, the microfluidic hardware and image processing software necessary to monitor fluorescence kinetics in droplets was developed using surfactant to control wetting. Subsequently surfactant was replaced by permanent surface modification reported in **Chapter 5**. It was limited to hexadecane as continuous phase but allowed first comparison with benchmark experiments using heavy metals. Next the microfluidic platform was further refined to create logarithmic concentration gradient enabling complete dose response curves for a more quantitative analysis of toxicity and detailed analysis of the resazurin kinetics with respect to initial concentration and bacterial density as reported in **Chapter 6**. Finally, surface modification was expanded to use fluorocarbons as continuous phase thus permitting testing of less polar organic compounds as detailed in **Chapter 7**. To help in the conception of the microfluidic platform, the resazurin assay was also separately and concurrently studied on microplates by Dr. Chen

Jianlin who also performed bacterial growth and methane production benchmark experiments as indicated in subsequent chapters.

3.2 Selection of Cell Viability Assay

Most commonly used cell viability assays that were considered are shown in **Table 3-1**. The MTT reductase assay is included because of its historical widespread use. However, detection by absorbance makes it ill-suited for sensitive detection in microfluidic where optical paths are typically 100 times shorter than on microplates. Furthermore, its major disadvantages, namely its toxicity and the need of secondary reagent to solubilize the formazan product are conveniently avoided by using resazurin. The resazurin assay also allows fluorescence readout which make it more sensitive and is important for rapid detection at sub-optimal incubation time. The ATP assay is typically regarded as the most sensitive, but because it takes time for the residual ATP to dissipate in non-viable cells, long pre-incubation time are required. The ATP assay also requires the presence of oxygen which make it complicated in microfluidic format, especially when pre-incubation is done under anoxic conditions. Fluorescein diacetate (FDAc) is an equivalently inexpensive alternative compared to resazurin but is subject so self-quenching [1] and photobleaching [2]. Lastly, the protease substrate glycyl-phenylalanyl-amino-fluorocoumerin (GF-AFC) is reported to produce detectable signal faster than resazurin under similar conditions. Although protease activity is expected in bacteria, literature data are primarily available for eukaryotic cells. It is also thousand times more expensive than resazurin which, despite the small volumes used in microfluidic, makes it unsuitable for continuous operations. In conclusion, the resazurin assay is well proven, fast and economical, hence was the most suitable for this initial work. Nevertheless GF-AFC and FDAc should still be considered in the future.

Table 3-1 Comparison of major cell viability assays [3-5].

Reagent	Principle	Detection	Advantages	Disadvantages
MTT	Reductase activity	Absorbance	Widespread acceptance	Endpoint, multistep protocol
ATP	ATP quantification	Luminescence	Sensitivity, widespread acceptance	Endpoint, long pre-incubation, require oxygen
Resazurin	Reductase activity	Fluorescence/absorbance	Simple, Inexpensive	Possible fluorescence interference
Fluorescein diacetate (FDAC)	Esterase activity	Fluorescence	Simple, Inexpensive	Photo-stability, overlapping abs/em peaks
Glycylphenylalanyl-aminofluorocoumerin (GF-AFC)	Protease activity	Fluorescence	Fast, non-toxic	Expensive, lack of data available for bacteria

3.2.1 Model Organisms

The resazurin assay can potentially be applied to any organism and their combinations. As early warning for anaerobic reactor both single strain and actual sludge could be used with distinct advantages. Good reproducibility can be expected from pure strains whereas sludge would give a tailored assessment of the reactor it was sampled from. One caveat of the later is that, since resazurin is not specific to the organism, inhibition of a small, but essential subpopulation such as archaea could be masked by the rest. Complications from granulation and flocs formation in real sludge are also to be expected. Consequently, pure culture was chosen for this initial work. Methanogenic archaea would be an ideal surrogate for anaerobic sludge as it is often regarded as the most susceptible to toxicants and represents the ultimate step in the digestion process. Unfortunately, archaea is notoriously difficult to culture and extremely sensitive to oxygen contamination [6]. At this early stage, *Enterococcus faecalis* was chosen because it is abundant in anaerobic digestion community [7], is easily accessible to many laboratories, and is responsible for the hydrolysis step

within anaerobic bioreactors. Although it is a facultative anaerobe, it switches to fermentation or anaerobic respiration when grown under anoxic conditions.

In the broader context of wastewater testing, the governmental requirements for drinking water are typically more stringent than what can be handled by a bioreactor, hence more sensitive organisms would be appropriate. Some bacteria from marine origin like the *Vibrio fischeri* at the heart of the Microtox bioassay are very sensitive towards certain toxicants but sometimes criticized for their relevancy to humans. Yeast on the other hand bears some similarities with mammalian cells, is inexpensive to culture and simple to maintain. Yeast has been genetically re-engineered to produce bioluminescence in response to pollutants but similarly to prokaryotes, several hours incubation are needed [8]. For this reason, yeast was chosen as a potentially more sensitive reporter with different relevancy.

3.2.2 Model Toxicants

In the initial stage a potent toxicant should be used to ensure that a response can be observed under sub-optimal conditions (bacterial density, incubation time, resazurin concentration, etc.). Pentachlorophenol (PCP) was chosen as model organic pollutants because it is the most toxic among chlorophenols. It is harmful to human and is listed as priority pollutants by the U.S. Environmental Protection Agency (U.S. EPA). Chlorophenols are also highly persistent and therefore problematic for biological wastewater treatment.

With an octanol/water logP of 2 [9], PCP is expected to be extracted by the oil phase in the droplet format. Albeit PCP being a stringent model to make sure the oil phase with the least interference is selected, heavy metals are another good model to avoid dealing with this obstacle in the early stage. Heavy metals are non-biodegradable and therefore also problematic in wastewater treatments. Small amount of certain heavy metals such as Zn, Ni, Fe, Cu, Co are essential to cells but also inhibitory at high concentration which ideally should be reflected by the resazurin assay.

3.2.3 Analysis of Resazurin Kinetics

Resazurin is first irreversibly reduced to fluorescent resorufin and subsequently, reversibly reduced to non-fluorescent di-hydroresorufin. Under anoxic conditions, the second reduction to di-hydroresorufin is irreversible. Assuming that the initial resazurin concentration $[A]_0 \ll K_m$, K_m being the Michaelis constant for the enzymes involved [10], the resazurin reduction kinetics can be described by two sequential pseudo first order reactions [11]. The resorufin concentration $[B]$ as function of time is given by:

$$[B] = [A]_0 \frac{K_1}{K_2 - K_1} (e^{-K_1 t} - e^{-K_2 t}) \quad (\text{eq. 3.1})$$

Where $K_1 = k_1[E]$, $K_2 = k_2[E]$ and $[E]$ is the enzyme concentration assuming the same enzyme is involved in both steps. As illustrated in **Figure 3.1** the fluorescence progress curve reaches a maximum intensity before dropping back. In conventional protocols, the fluorescence intensity is only measured once after a fixed incubation time which can lead to erroneous results if the linear region has not been determined by preliminary experiments [12]. Secondary reduction can be clearly identified in progress curves and model can be fitted to determine the rate constants. Accuracy of the fitting is however limited by the timescale available. In the example below, when toxicant is added the decrease in fluorescence is not visible anymore on the timescale available, resulting in overparameterized fitting. A more generic approach is to neglect the second conversion for short timescale and to look at the initial rate by fitting a linear equation to the initial portion of the data. To account for large variation in rates, the time window can be adjusted to where 5 to 10% of the starting resazurin has been converted. Furthermore, estimation of initial rate is more robust than single time point because it averages multiple measurements and is independent of background fluorescence.

Figure 3.1 Theoretical example of progression curves for resazurin reduction based on **Equation (3.1)** with 10 μM starting resazurin concentration. Magnification of the first 5 minutes is shown in inset. Curves correspond, from top to bottom, to normal rate without any toxicant and increasing levels of inhibition.

3.2.4 Complementary Laboratory Methods to Assess Toxicity and Comparison Metric

Several comparisons can be made to validate our method. First the resazurin assay was performed on microplates to investigate the effect of the oil phase, continuous mixing, time resolution to prepare the samples, etc. Viability assays measure changes cellular metabolism but do not necessarily indicate if organisms are still able to grow. Comparison with proliferation can be made monitoring bacterial growth through optical density. Parameters such as lag time, growth rate or maximal population can be extracted from growth curves [13]. Relevancy of the sensor output to anaerobic sludge should also be addressed. Among the many parameters indicative of an anaerobic digester performance, the methane production is the most prevalent and was selected here as it is the final step in

anaerobic digestion and reflect how much energy can be produced in industrial settings [14]. Finally, our toxicity assessment can be compared with environmental regulations such as Singapore¹ or U.S.² EPA to determine its practicality for other applications (discharge regulation, drinking water, etc.).

Although the nomenclature and signal direction might differ depending on the method used, when the response is plotted versus the log of the concentration, the response typically follow a sigmoidal curve with 2 plateaus at no inhibition and complete inhibition. If symmetric, it can be fitted with a 4-parameter logistic function:

$$y(x) = Max - \frac{Max - Min}{1 + \left(\frac{x}{EC50}\right)^{Hill}} \quad (\text{eq. 3.2})$$

The denomination and definitions of derived parameters adopted in this thesis are illustrated in **Figure 3.2**. The limit of detection (LOD) is determined from the standard deviation of the blank and sample according to ref. [15]. Threshold and saturation concentrations, also referred in proliferation assays as non-inhibitory concentration (NIC) and minimum inhibitory concentration (MIC) respectively, follow the definition in ref. [16]. Depending on context, the midpoint is interchangeably referred to as half inhibitory concentration (IC50) or half effective concentration (EC50). For sake of constancy, EC50 will be used henceforward. More importantly, asymptotic values do not always reach the level of the positive or negative controls. In this case a distinction is made between relative and absolute EC50 when the fitted asymptotes or control values are used respectively [17]. EC50 and LOD must also be put in perspective with response time which are often negatively correlated. Finally, viability, proliferation or cytotoxicity assays should be compared with care and are not always distinct. For instance, in viability assays with toxicant pre-incubation longer than the cell doubling time, the response can be due to a

¹ <http://www.nea.gov.sg/anti-pollution-radiation-protection/water-pollution-control/allowable-limits>

² <https://safewater.zendesk.com/hc/en-us/categories/201454937-Fact-Sheets>

combination of difference in metabolic activity and cell number because of proliferation in untreated control.

Figure 3.2 Typical sigmoidal response of analytical methods. Upper and lower limits can be reversed depending on the nature of the method used.

3.3 Microfluidic Design and Considerations

Transferring the resazurin assay in microfluidic platform is not straight forward because of laminar flow at low Reynolds number characteristic of microchannel. As shown in **Figure 3.3**, reagents injected in microchannel are only mixed by diffusion. Furthermore, what is not apparent from the figure is that flow velocity is maximal in the centre and zero near the walls which results in temporal dispersion. In other words, even if resazurin could be perfectly mixed with bacteria, the incubation time would be several times shorter in the

centre of the channel than on the sides. Segmented flow conveniently solves both, mixing and temporal dispersion issues but requires extra steps to control surface wetting and minimize partition into the oil phase as discussed below. Compartmentalization into droplets also allows different reagents mixtures, including positive and negative controls to be tested simultaneously.

Figure 3.3 Comparison between laminar flow and segmented flow (water slugs in continuous oil phase). Channel is 700 μm wide and 100 μm deep.

Droplets volume can vary from microliters down to femtoliters depending on channel size. While smaller volumes means less reagents are used, the size and density of microorganisms have to be considered as well. At the lower end of the spectrum, distribution of droplets with single cell or no cell can be obtained but are out of the scope of this thesis [18]. For our purpose a minimum of hundred cells per droplet would ensure the response is representative of the entire population. If mixed culture such as sludge are considered this number should be increased accordingly. Channel dimensions are also limited by the fabrication method and imaging technique as addressed in the following

sections. Given a channel cross-section of $100\ \mu\text{m} \times 700\ \mu\text{m}$, a droplet $1.5\ \text{mm}$ long has an approximate volume of $100\ \text{nL}$. Maximum cell concentration in stationary phase are in the order of 10^7 and $10^9\ \text{cell/mL}$ for yeast and bacteria respectively, which would result in 10^3 to 10^5 cells per droplet, giving enough margin to use cell in exponential phase as well.

3.3.1 Distribution Coefficient Between Aqueous Droplets and the Continuous Phase

In the absence of surfactant, the main mechanism for undesirable extraction and exchange of solute between droplets is through partition into the oil phase. Reagents and bacterial culture are highly soluble in water, but problems arise when non-polar pollutants such as our model for organic toxicant PCP need to be tested. Measurements of partition coefficients can predict this behaviour and standard methods are described in OECD guidelines 107 and 123 [19-20]. Two major modifications had to be made for our purpose. First, octanol-water partition is standard to report lipophilicity and make comparison across laboratories. Since we are interested in the effect of the oil phase, in particular the difference between hydrocarbons and fluorocarbons, coefficients were measured with the continuous phases instead of octanol. Second, water was at physiological pH which means PCP can be partially ionized (pK_a of 4.8) and is more relevant here since that is how it ends up in wastewater. Measurements therefore correspond to the distribution coefficient D [21] defined as:

$$D = \frac{[\text{Solute}]_{\text{oil}}}{[\text{Solute}]_{\text{water}}^{\text{ionized}} + [\text{Solute}]_{\text{water}}^{\text{non-ionized}}} \quad (\text{eq. 3.3})$$

Distribution coefficients are difficult to predict, however relative solubility in the oil phase can be used to compare liquids. Solubility in non-ionized form (i.e. in oil) can be estimated with Hansen solubility parameters $\delta_d, \delta_p, \delta_h$ that represents the cohesive energy decomposed in dispersive, polar, and hydrogen-bonding components respectively [22]. Solubility of 2 molecules (e.g. oil and PCP) can be ranked according to their Hansen distance:

$$Ra^2 = 4(\delta_{d1} - \delta_{d2})^2 + (\delta_{p1} - \delta_{p2})^2 + (\delta_{h1} - \delta_{h2})^2 \quad (\text{eq. 3.4})$$

3.3.2 Choice of Material for Microfluidic Chip

Poly(methyl methacrylate) was chosen as the best candidate for the design of microfluidic due to its optimal material properties, as outlined in **Table 3-2**. However, several other materials were considered. Polydimethylsiloxane (PDMS) has been the go to material for prototyping microfluidic in the past two decades. It is however not suitable for our purpose because of its large gas permeability that prevent anoxic conditions and incompatibility with alkanes. Besides physical parameters, the fabrication and scale-up manufacturing was also taken into consideration. A quick turnaround is helpful in the development stage to make rapid adjustments which is why PDMS has become so popular. A common criticism towards PDMS is that it does not lend itself to production scale-up which could be partly responsible for the lack of commercial transfer of microfluidic technologies [23]. Some of the materials listed in **Table 3-2** can be processed by different methods suitable to both, prototyping and production, thus possibly shortening future technology transfer.

Table 3-2 Comparison candidate materials for droplet microfluidic. More general comparisons can be found in [24-25].

Material*	PMMA	PDMS	PC	PS	COC	FEP	TFE/PDD
Transparency	UV-VIS	UV-VIS	VIS	VIS	UV-VIS	UV-VIS **	UV-VIS
Low gas permeability	yes	no	yes	yes	yes	yes	no
Hydrocarbon compatibility	yes	no	yes	no	no	yes	yes
Fluorocarbon compatibility	yes	yes	yes	yes	yes	yes	no
Need surface modification	yes	yes	yes	yes	yes	no	no
Prototyping***	lit, mill	lit	mill	mill	mill	?	?
Replica molding	yes	no	yes	yes	yes	yes	yes
Material cost ~ (\$/kg)	3	150	3	3	25	50	100 000

* Polydimethylsiloxane (PDMS), Poly(methyl methacrylate) (PMMA), polycarbonate (PC), polystyrene (PS), cyclic olephyn copolymer (COC), fluorinated ethylene propylene (FEP), tetrafluoroethylene-perfluorodimethyldioxole-fluorocopolymer (TFE/PDD)

** scattering due to crystallinity, only suitable for thin films

*** photolithography (lit), micromilling (mill)

Fluoropolymers have a major advantage in that wetting by the continuous oil phase does not require surface modification. Unfortunately, they tend to be crystalline thus limiting optical applications. Due to its cost, TFE/PDD is not meant for bulk use but illustrates the point that amorphous fluoropolymers have better transparency at the cost increased solubility in fluorocarbons. Micromilling if available is a fast prototyping technique but generally lithography results in better surface finish. Overall, PMMA was chosen as the best candidate that fulfils the priorities of solvent compatibility, transparency and rapid prototyping.

3.3.3 PMMA Microfluidic Fabrication Methods

PMMA is one of the most versatile material in terms of fabrication technique. Deep UV etching and micromilling were used in this work due to their respective advantages. PMMA has long been used as high resolution photoresist for deep UV and e-beam lithography and recently Haiducu et al. have shown that the same process can be applied to PMMA sheets to create channel up to 100 μm deep [26]. We further simplified their procedure by replacing the intermediate metal mask with laminated photoresist as illustrated in **Figure 3.4**. Exposure to UV is typically done overnight to obtain the maximum depth and after development in isopropanol. Channels is perfectly transparent but polymer near the surface still has a lower molecular weight than the bulk resulting in poorer solvent resistance (e.g. ethanol). Micromilling [27] on the other hand only takes 1-2 hours in total but tooling marks remains which can be a problem when channel is filled with liquid of largely different refractive index (cf. **Chapter 4**). Channel depth sometime varies more, depending on the thickness consistency of the starting PMMA sheet. In the last step, bonding can be performed by chloroform vapours which simultaneously reduces surface roughness [28]. Finally, other methods were considered but not selected. Laser ablation is fast but was not used here because of its poor optical finish. Films lamination with adhesives was ruled out as well because of the resulting heterogenous surface chemistry of the channel walls which would alter wetting properties.

Figure 3.4 (A) Scheme for etching of microfluidic channels on PMMA slides. (B) PMMA etching depth versus 254 nm UV exposure time.

3.4 Surface Modification of Microchannel

Several approaches are possible to ensure preferential wetting by the continuous phase. Dynamic coating with surfactant was used in the first stage due to its simplicity and was later replaced by permanent modification. Bulk modification of the polymer was not considered as a viable approach because it would compromise other properties such as transparency or solvent resistance as mentioned in previous section. Plasma assisted surface modification is versatile but for microfluidic, it must be done before bonding because of diffusion limitations in enclosed channel. Hydrophobic modification prevents bonding of the chip, which means extra masking steps are required. Despite this complication, the method remains attractive because it is mostly substrate independent and existing protocols could potentially be used [29]. Modification by wet chemistry after bonding was also tried. After several unsuccessful attempts through routes specific to PMMA, a method based on silica nanoparticles (SiNPs) primer described in **Chapter 5** was developed. During this process, the modification was first assessed by dynamic contact angles measurement because of its simplicity and speed. Most promising methods were applied inside microchannel and further characterized by atomic force microscopy (AFM) and scanning electron microscopy (SEM) where appropriate.

3.4.1 Controlling Wetting with Surfactants

Figure 3.5 Partial wetting regime in pristine PMMA channels and droplets stabilization with surfactant.

As shown on **Figure 3.5**, with water and hexadecane, partial wetting regime is observed on pristine PMMA channel. When bacteria culture is added, transition to oil in water regime is favoured because of surface active molecules present in the medium. It can easily be prevented by adding oil soluble surfactant in the hexadecane phase. While surfactants prevent wetting of the wall by the dispersed phase, it also prevents spontaneous coalescence between droplets and promote transfer of solute between droplets through micelles as illustrated in **Figure 3.6**. No optimal surfactant concentration could be found to prevent wetting by the dispersed phase while allowing reliable injection of reagents into existing droplets. As a temporary solution until permanent surface modification was developed, the channel was flushed with 1% Span 80 before every use. When removed from the continuous phase, surfactant remains temporarily adsorbed on the walls thus allowing droplets coalescence for about 30 minutes of operation.

Figure 3.6 Role of surfactants in droplet microfluidic.

3.4.2 Dynamic Contact Angle

Contact angle (CA) is a rapid method to quantitatively assess wettability. On chemically homogenous and perfectly smooth surface, the contact angle θ of a drop is described by the Young equation.

$$\gamma_{SL} + \gamma_{LV} \cos \theta = \gamma_{SV} \quad (\text{eq. 3.5})$$

Where γ_{SL} , γ_{LV} , γ_{SV} are the interfacial tensions between the solid and liquid, between the liquid and gas and between the solid and gas respectively. In practice, some degree of hysteresis is always observed between the advancing and receding front of a droplet. Depending on how the droplet was deposited, the static contact angle lies somewhere between a minimum receding angle and a maximum advancing angle, generally closer to the advancing angle. Whyman et al. derived a thermodynamic model describing the contact angle hysteresis (CAH) [30]:

$$CAH = \theta_{adv} - \theta_{rec} = \left(\frac{8U}{\gamma_{LV}R_0} \right)^{1/2} \frac{(1 - \cos \theta^*)^{1/12} (2 + \cos \theta^*)^{2/3}}{2^{1/3} (1 + \cos \theta^*)^{1/4}} \quad (\text{eq. 3.6})$$

Where U is the height of the potential barrier connected with the motion of the triple line along a substrate, R_0 is the initial radius of the spherical drop before deposition on the substrate and θ^* is the apparent contact angle at equilibrium. Other models considering dynamic phenomenon have also been proposed [31].

On rough or chemically heterogeneous surfaces, the hysteresis effect is more pronounced and it is preferable to measure the advancing and receding angles [32]. It can be achieved by the dynamic sessile drop method where the volume of the drop is gradually increased or decreased until the maximum or minimum contact angle is reached respectively. This method is however not recommended for high CA because interactions with the needle can significantly deform the drop profile. In that case, the tilt plate method illustrated in **Figure 3.7A** is more suitable but requires a goniometer with a tilting stage.

Figure 3.7 Contact angle measurement with the tilt plate method (A) Graphical representation of the advancing and receding CAs and the tilt angle (B) Example of record for a water drop on pristine PMMA.

On heterogenous surfaces, contact lines sometimes move in irregular fashion. To ensure repeatable analysis, the advancing and receding CAs are taken at the point where the receding front starts to move, which can be determined from the maximum base diameter of the drop as shown in **Figure 3.7B**. To be consistent, the drop volume should be the same for all substrates and needed to be at least $\sim 20 \mu\text{L}$ for the drop to slide on all our samples. Because of the asymmetry of drop, the contour cannot be analysed with circle or ellipses fitting like in the sessile drop method, instead the tangents were obtained by polynomial fitting. CA in air is good to rapidly measure trends between different surface modification methods but for droplet microfluidic, it is more relevant to replace air with oil. In that case, a high CA ($\ll 90^\circ$) and low CA hysteresis ($< 10^\circ$) warranted further test in actual microchannel. When dealing with textured surfaces, one also must consider transitions between low hysteresis in Cassie wetting state and high hysteresis in the Wenzel state which is often the thermodynamically stable one. In microchannel, the situation is further complicated by capillary pressure and the presence of flow rate dependent lubricating layer that are not accounted for in CA measurements and are further discussed in **Chapter 5**.

3.4.3 Atomic Force Microscopy (AFM)

Wetting is controlled by surface chemistry and morphology and while some topographical information can be deduced from CA measurements, AFM provide a direct quantification of surface roughness as well as coverage in the case of nanoparticle coatings. Phase analysis can also be used for chemical mapping based on local difference in stiffness and adhesion with the tip, even on surfaces of uniform roughness. To extend the tip lifetime, images were acquired using tapping mode in attractive regime, also known as non-contact mode. Mode hopping is a common artifact of this method where the tip substrates interactions intermittently switches between attractive and repulsive forces. It can be mitigated by driving the tip above its resonant frequency and adjusting several other parameters, but ultimately it cannot be completely avoided on certain samples. **Figure 3.8** illustrates such situation where the combination of very dissimilar materials (silica, PMMA) and steep height variations result in transition into repulsive mode upon scanning up steps

(i.e. phase $< 90^\circ$). Another artifact to keep in mind is that the images obtained by AFM are the convolution of the tip with the substrate and lateral dimensions can be significantly overestimated for protrusions (e.g. a < 10 nm tip radius scanning 25 nm nanoparticles) or underestimated for holes. Therefore, height signal just be used to measure particle sizes.

3.4.4 Field Emission Scanning Electron Microscopy (FESEM)

SEM was also used to analyse surface created by nanoparticles coating as well as plasma assisted modification. Compared to AFM, SEM allows rapid scan at multiple scales from mm down to nm. This is useful to ensure that lateral surface features larger than a few microns are not missed by AFM and is also much faster to estimate surface coverage by nanoparticles over large areas. Surface roughness can also be quantified by stereo-SEM using multiple image acquisition on the same spot using different tilt angles if the sample do not deteriorate. FESEM had to be used to resolve the small size of the nanoparticles used in this study ~ 10 -100 nm because field-emission cathodes generate a more focused electron beam than conventional thermionic emitters. To prevent excess charge building up, samples were coated with platinum. Platinum was used instead of gold because of its smaller grain size and to ensure it wouldn't be mistaken with SiNPs, pristine sample with the same coating were imaged as control using a scratch to help focusing.

Figure 3.8 Example of AFM height and phase images of silica nanoparticles on PMMA. Lines were scanned from left to right.

3.5 Conclusion

This chapter provided the rationale for selecting segmented-flow microfluidic and resazurin as viability indicator. A case was also made to measure time course fluorescence intensity instead of single timepoint. The next chapter describes in details how the microfluidic platform was designed to achieve these goals.

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Chapter 4

Microfluidic Light Guide Fabrication and Design Rationale Towards Real-Time Viability Analysis

This chapter introduces the microfluidic setup and complementing software that was developed and used thorough this thesis. The physical setup includes a PMMA microfluidic chip to generate droplets with addressable content, LED-based fluorescence excitation and a color camera to measure both, scattering and fluorescence signals of aqueous droplets in a continuous oil phase. Main steps of video processing to extract resazurin kinetics data are detailed, including background estimation, droplet segmentation and tracking as well as fluorescence normalization.

4.1 Introduction

A custom setup and video analysis software was developed to monitor the conversion of resazurin to fluorescent resorufin in microfluidic droplet format. Given the secondary conversion of the chosen viability indicator to non-fluorescent dihydro-resorufin and non-linearity at high bacterial density, time course measurements were preferred to a single measurement after a predetermined incubation period. The device also had to operate in continuous fashion for real-time monitoring. Injection of bacteria and resazurin needed to be separate to prevent cross-contamination and ensure bacteria are mixed with toxicant before adding resazurin. Variation of the sample concentration was also required for control samples and dose-response relationship. To do so droplets were generated and tracked along the incubation channel in continuous fashion with a camera as depicted in **Figure 4.1**. The algorithm runs in real time, thus minimizing the overall response time of the sensor but also provides a mean to control the pumps via video feedback based on parameters such as the number of droplets generated, their size, fluorescence intensity, etc.

Figure 4.1 (A) Microfluidic platform overview. Droplets containing adjustable toxicant concentration are formed at the first T-junction. Bacteria and resazurin are sequentially injected in existing droplets. Before each injection, the droplet content is homogenized by flowing through meanders. Finally, fluorescence intensity of each droplet is recorded for 5 minutes until they exit the chip. Fluorescence excitation is provided by total internal reflection from 56 LEDs distributed around the chip. (B) Train of droplet containing bacteria and resazurin without toxicant.

Fluorescence intensity increases when resazurin is converted by bacteria to resorufin along the incubation channel (flow in zig-zag pattern from top to bottom).

4.2 Experimental Methods

4.2.1 Materials and Chemicals

Hexadecane, 99% was purchased from Alfa Aesar, USA Span 80 was purchased from Wako Pure Chemical Industries Ltd, Japan. Fluorocarbons (FC) FC-40, FC-43, FC-3283, FC-770 and hydrofluoroether (HFE) HFE-7500 were purchased from 3M, Singapore. Resorufin (7-Hydroxy-3H-phenoxazin-3-one sodium salt) was purchased from Sigma Aldrich, Singapore. Deionized water was used in all experiments. PMMA sheets 3 mm thick were obtained from Marga Cipta, Indonesia.

4.2.2 PMMA Microfluidic Chip Fabrication by Deep UV Etching

PMMA microfluidic chips were fabricated by a simplification of the procedure described by Haiducu et al. [1] where sputtering of an intermediate metal mask was avoided as shown in **Figure 3.4**. Briefly, a dry negative photoresist film (Riston FX515, DuPont) was laminated onto a PMMA slide and patterned by UV at 360 nm using an inkjet printed mask. Once developed the photoresist was used as a mask for exposing PMMA to UV at 254 nm for 12 hours. Exposed PMMA and the remaining photoresist were dissolved by sonication in 70% IPA at 20 °C, resulting in channels approximately 100 µm deep. One mm diameter ports were drilled and the chip was closed by thermally bonding a pristine PMMA slide at 27 bar and 80 °C for 30 minutes followed by ~200 mbar at 110 °C for one hour. Finally, the edges were sanded with 320 grit sandpaper to act as light diffuser.

4.2.3 Microfluidic Device Operation and in-situ Fluorescence Recording

Figure 4.2 Custom microfluidic setup. (A) 3D printed assembly including LEDs excitation for fluorescence measurements, camera for tracking droplet and recording fluorescence signals and a heater for bacterial viability experiments at 37 °C. (B) Photo of a 6 cm x 6 cm PMMA microfluidic chip surround by 56 LEDs in the bottom part of the holder. (C) LED fluorescence excitation through the PMMA chip acting as a lightguide to avoid direct line of sight with camera.

The PMMA chip with outer dimensions of 6 cm x 6 cm was placed in a custom holder 3D printed from black ABS as depicted in **Figure 4.2**. Fluidic connections were made with 1/32'' ID FEP tubing (EW-06406-60, Cole-Parmer) and flangeless fittings (P-200, P-288, Upchurch scientific). Fluorescence excitation was achieved by total internal reflection from 56 LEDs at 530 nm (151033GS03000, Würth Elektronik) surrounding the chip and were operated at a current of 5 mA. The assembly was maintained 37 °C by a Pelletier element. Dual syringe pumps (Mitos Duo XS-Pump, Dolomite) and two syringe pumps (Pump 11 Elite, Harvard Apparatus) were used for the aqueous inlets with a combined flow rate of 1 $\mu\text{L min}^{-1}$. Hexadecane was delivered from a pressurized reservoir (~1 bar), and the flow

was adjusted with a needle valve (P-445, Upchurch scientific) to maintain the distance between droplets of approximately twice the droplet length.

To ensure wetting of the channel walls by the oil phase, surfactant was used in preliminary tests and subsequently replaced by permanent surface modification as detailed in **Chapter 5** and **Chapter 7** for hexadecane and fluorocarbons respectively. Microfluidic chips were first filled with hexadecane containing 1% (w/w) of span 80. The surfactant concentration was then reduced to 0.05% (w/w) before generating droplets. This procedure allowed droplet generation and injection into existing droplets for about an hour before flushing again with 1% (w/w) span 80.

4.2.4 Video Acquisition and C++ Software

Images were acquired using a digital camera (acA1300-60gc, Basler) with a 12 mm focal length lens (M1214-MP2, Computar) and processed on a desktop computer (Intel core i5-3470, 4 GB DDR3 1600M HzRAM). A C++ software was developed in-house using the OpenCV library [2] to simultaneously track and record fluorescence intensities of all droplets present in the chip as well as to control the pumps. The algorithm is presented and discussed in detail in the next sections.

4.3 Results and Discussion

Conventional fluorescence detection in droplet microfluidic are typically carried out at a single point with a focused laser beam or within the small field of view of an optical microscope. In this work, parallelization of droplet monitoring was achieved by choosing a lower magnification lens to cover the entire incubation channel (5 cm x 5 cm). Large field of view comes at the cost of lower sensitivity [3], but is not a primary concern here because long integration time can be used at low frame rate (up to 100 ms at 10 fps) and also because resorufin is used at μM concentration and has a high quantum yield of 0.74 [4]. For excitation, LEDs are an inexpensive option and excitation by total internal reflections avoids direct reflection into the camera as illustrated in **Figure 4.2C**. The acceptance angle of a planar waveguide is given by $\theta = \arccos(n_{\text{cladding}}/n_{\text{core}})$ [5]. For a

PMMA chip surrounded by air, $\theta = 47.8^\circ$ which is much larger than 15° , the half viewing angle of the LEDs. Since the chip is 6 mm thick, twice the diameter of the LEDs, no additional optic is required and the alignment is trivial. Unlike total internal reflection microscopy [6] where only molecules in the evanescent field are excited, when the microfluidic chip itself is used as the light guide, the entire volume of the droplets is excited [7]. With this configuration, only the excitation light that is scattered at the oil-water and oil-PMMA interfaces reaches the camera and is easily separated from fluorescent signal using a color camera as illustrated in **Figure 4.3**. Consequently, droplets can be segmented and tracked using scattering signal from the green layer or all 3 layers converted to grayscale, regardless of their fluorescence intensity while fluorescence intensity is measured from the red layer.

Figure 4.3 Resorufin absorption and emission spectra in comparison to the green LEDs used for excitation and the spectral response of the color camera.

The overall workflow to extract fluorescence data over time for each droplet is illustrated in **Figure 4.4**. The general approach is typical of video surveillance processing and therefore most of the functions needed were available in the OpenCV library [2]. In the context of droplet microfluidic, details for different tasks such as size distribution analysis,

cell encapsulation efficiency, tracking of packed droplets, etc. have already been discussed by Basu [8]. The next three subsections address aspects specific to the application at hand with emphasis on algorithms suitable for real time processing of high definition stream (1280x1024 px²).

Figure 4.4 Video processing to extract droplet information. (A) Raw color image acquired by the camera. Four aqueous inlets are labeled in white. (B) Fluorescence intensity data extracted from the red layer after background subtraction. (C) Droplet tracking from color image after background subtraction, thresholding and contour detection (arbitrary color labeling). (D) Fluorescence intensity recorded for one droplet.

4.3.1 Background Image Estimation

To detect the droplets (foreground) an image of the channel filled with oil but without droplets (background) need to be subtracted. Instead of saving a background image once before generating droplets, a more robust and convenient way is to continuously estimate a background model. For initial experiments, the “mixture of Gaussian” (MoG) [9-10] algorithm implemented in openCV was used to extract the background. While it can update

the background dynamically, initialization still required a droplet free channel and when the oil/droplet ratio was close to unity, choosing the right refresh rate and threshold was difficult. Since droplets fill less than 50% of the channel (oil/droplet ratio > 1) taking the median intensity for each pixel over the last 10-100 frames results in a very robust background estimation but is computationally and memory wise too expensive [11].

Instead a hybrid algorithm between a moving average and temporal median was developed. Each pixel of the background image is updated by a moving average with pixel from the last acquired frame only if it is brighter (darker) than the background and has been brighter (darker) more than 50% of the time. It can be efficiently implemented with two matrices 1) COUNT: % of time the current pixel intensity is larger than the background and 2) BCKG: estimated background image. Both are updated by a moving average after each frame is acquired as described in pseudo code in **Table 4-1**.

Table 4-1 Pseudo code for real-time background estimation of droplet microfluidic videos. Hybrid algorithm between a moving median and moving average.

Description	Pseudo-code
0. For the first call, Initialize background with current image	BCKG = CURRENT
1. Compare current image to background	COMP = CURRENT > BCKG
2. Calculate a mask where the background needs to be updated: Which is the combination of both conditions:	MASK = !(COMP xor COUNT>0.5)
A. Current intensity is greater than background and has been for more than 50% of the time	A. COMP & COUNT > 0.5
B. Current intensity is lower than background and has been for more than 50% of the time	B. !COMP & COUNT ≤ 0.5
3. Update background (h=history length)	BCKG = (h-1)/h BCKG + 1/h CURRENT where MASK == true
4. Update COUNT (h=history length)	COUNT = (h-1)/h COUNT + 1/h COMP
5. Repeat from 1 with next frame.

Figure 4.5 Example of background estimation from a video sequence of moving droplets using a hybrid algorithm between a moving average and moving median. More frames are needed for initialization compared to a moving median but it is nearly 3 orders of magnitude faster to process.

The pseudo code was implemented in C++ and run at least as fast as OpenCV MoG2. This method only requires tuning one parameter: the history length (versus 4) and can be initialized with foreground objects already present. A video sequence of moving droplets was recorded at 10 fps to compare the background estimation from our algorithm with an actual median estimate, both with a 21 frames window. As shown in **Figure 4.5** the hybrid algorithm takes more frames than a median to initialize due to the moving average component but eventually converges to the same background estimation. More importantly, it only takes 12.7 milliseconds per frame to compute compared to about 5 seconds for a moving median on the same computer. The complexity is $O(1)$, meaning that the execution

time remains constant with the history length, whereas the median is $O(n)$ at best and linearly increases with history length.

4.3.2 Droplets Segmentation and Tracking

Figure 4.6 Example of droplet contour detection. The background image of the channel filled with oil is subtracted to the acquired image and a threshold is applied. Even non-fluorescent droplets are visible due to scattering of excitation light (green layer) at the oil-water interface. While trivial for hexadecane, in case of fluorocarbons more light is scattered at the PMMA channel boundary because of refractive index mismatch. The threshold level cannot be lowered to isolate the droplet contour without picking up noise. A simple box blur reduces the noise level while maintaining the intensity of the droplet contours.

Once obtained, the background model is subtracted to every new frame and a threshold is applied to create a binary image of the droplets' boundary as illustrated in **Figure 4.6**. Segmentation is greatly affected by the refractive index of the continuous phase. Compared

to hexadecane, fluorocarbons have a refractive index closer to water (**Table 4-2**), making the droplets' boundary fainter because of decreased scattering at the water/oil interface. Scattering at the channel edges (oil/PMMA interface) is on the other hand increased and tend to saturate the camera detector. Using the same threshold level as for hexadecane, the droplet boundary is incomplete and nearly impossible to track. Simply lowering the segmentation threshold introduces too much noise because the intensity of the droplet boundary is close to the background level. Unlike random noise however, droplets boundaries are spatially confined. This spatial information can be leveraged by applying a blur (2D box averaging window) before thresholding. It reduces the noise which is averaged out with “empty” neighbouring pixels while droplet boundaries remain above the threshold. This extra step is more efficient than typical morphological processes applied to the binary image after thresholding and does not reduce the overall frame rate (~10 fps).

Table 4-2 Refractive indices of liquids and material used for droplet microfluidic

Water	1.33
Hexadecane	1.43
FC-40, FC-43, FC-3283, FC-770	1.27-1.29
HFE-7500	1.29
PMMA	1.49

After thresholding, contours of each disconnected geometric sample (i.e. droplet) is detected (**Figure 4.4C**). Its center of mass is used to estimate the droplet position while its area is indicative of the droplet volume. Droplet tracking is obtained by simply assigning each blob to the closest droplet in the previous frame. This approach works well when droplets move by a distance less than their interspace between two consecutive frames.

4.3.3 Fluorescence Intensity Normalization

To make the excitation light more uniform, 56 LEDs operated at low current (5 mA vs. 30 mA max) were distributed around the chip and the edges were sanded with 320 grit

sandpaper to act as light diffuser. As shown in **Figure 4.7**, it is still not perfectly uniform, but remaining intensity variations can be corrected if the intensity falls within the linear range of the camera sensor. Intensity variations are also due to lensing effect in the corner of the channel and fluctuation of the channel depth (i.e. optical path length). Spikes are observed because, the center of mass for long droplets (slugs) falls outside of the channel in the corners due to their concave shape.

To compensate for these variation, a normalization image can be obtained by either filling the channel with resorufin or accumulating the fluorescence traces of resorufin droplets in oil with a moving average and using the foreground objects as a mask. The latter is preferable because it can account for lensing effect at the droplets boundaries and differences in light coupling efficiency when an oil layer surrounds the droplet. Finally, a moving median filter (3-5 s) is applied to remove high frequency noise. After the normalization process, the detection range for resorufin was from 100 nM to approximately 10 μ M (camera saturation). The lower limit of detection was estimated from the standard deviation of the blank and low concentration samples [12].

Figure 4.7 (A) Fluorescence normalization image obtained by accumulating traces of resorufin droplets. (B) Example of data recorded for a single droplet. Intensity is normalized with respect to a fixed reference point in the left image.

4.3.4 Microfluidic layout Rationale

The microfluidic chip was designed to generate droplets with addressable mixture of 2 reagents (e.g. toxicant and diluent) followed by sequential injection of another 2 reagents at 20% each of the final droplet volume (e.g. bacteria, resazurin). The exact layout was optimized through several iterations and lead to the following choices. The channel depth (i.e. optical path length) was set to the maximum achievable depth by deep UV etching: 100 μm , to maximize the fluorescence signal. The width of the main channel was chosen as 700 μm which corresponds to 20 pixels on the camera. It ensures that fluorescence intensity at the centre of the droplets is not masked by scattering from the edges. The spacing between channels was also set to 700 μm and the total length was maximized by filling up the field of view of the camera. It resulted in a total length of around 90 cm, corresponding to 5 min incubation under typical operating conditions. The droplet length was kept as small as possible to reduce the mixing distance [13-14] (**Figure 4.8**) but had to be more than the channel width for repeatable injection and was set to 1400 μm (i.e. twice the channel width). The width of the generating T-junction was empirically adjusted to 300 μm (**Figure 4.9**) to achieve the desired droplet size and create an oil spacer approximately twice longer (after all injections). At higher oil ratio, the droplet size is almost independent of the total flow rate (i.e. incubation time) [15]. The width of the injection channel was set to the smallest width achievable with our fabrication method: 200 μm , to minimize cross contamination [16].

Figure 4.8 Mixing in droplets of different length: ~4 mm (top) and ~2 mm (bottom) long plugs. Resorufin is injected in water droplets and mixes as it goes through meanders. Smaller droplets are completely mixed after a shorter distance travelled.

Figure 4.9 Design of T-junctions for droplet generation and injection into existing droplets. Channel depth is 100 μm . Final droplet volume consists of 60% of the original droplet and 20% of 2 separate injections. Final oil to water ratio is approximately 2.

4.3.5 Control of Droplet Content

An advantage of using a bioassay in droplet microfluidic format over a conventional biosensor is that multiple control samples as well different concentrations of toxicant can be generated. Concentration gradients in fixed size droplets can be obtained by combining sample and diluent streams having a fixed total flow rate right before droplet generation [17-18]. For testing purposes, the fluorescent dye resorufin and DI water were injected at the first 2 aqueous inlets. Flow rates were automatically adjusted by the software after each droplet to create a linear and stepwise concentration profile. Video feedback, unlike time base flow rate control, ensures that the concentration profile remains the same regardless of the volume of the droplets which depends not only on the channel geometry, but also viscosity and flow rate of the oil phase. The average fluorescence intensity of each droplets is shown in **Figure 4.10**.

Figure 4.10 Average intensity of droplets with varying concentrations of resorufin obtained by varying the flow rates between two pumps (A) linearly and (B) in stepwise fashion. Error bars in (A) represent the standard deviation of 3 repeated cycles.

In both concentration profiles, deviation from targeted concentration is the highest at mid-range and suggests a small mismatch between the two pumps. Delays are also evident in the stepwise gradient where it takes 3 to 6 droplets to reach a concentration plateau. A delay of one droplet can be attributed to the camera feedback loop, the rest is due to flow rate stabilization time with syringe pumps. This is a limitation inherent to syringe pumps

and flow rates oscillations can be observed for up to hours depending on tubing used, trapped gas bubbles, etc [19-20]. For this reason, concentration gradients are typically limited to 2 orders of magnitude [21]. A practical implication for rapid transition between concentrations is that the first few droplets immediately after should be discarded from analysis.

Compared to syringe pumps, pressure driven flow stabilizes quickly, but controlling the flow rates is not as straight forward. A solution to generate concentration gradient spanning at least 3 orders of magnitude is presented in **Chapter 6**.

4.4 Conclusion

A complete microfluidic setup and C++ video processing software was developed to generate droplets with addressable content as well as monitor the kinetics of fluorescence products formation in 100 nL droplets. The design uses a camera to monitor the entire microfluidic chip allowing continuous and independent monitoring of 50 to 100 droplets as they flow along the channel. Fluorescence intensity of each droplet is recorded over a 5 minutes window with 0.1 second resolution and at a throughput of ~20 droplets per minutes, which can be adjusted by varying the flow rate. Thus, linear region of the curve can be clearly identified to determine initial velocities of resazurin reduction. This continuous approach along with small volumes involved in microfluidics allows using a traditional laboratory assay that is inherently robust due to multiple test and control samples as an online sensor. This initial work was done using surfactant as temporary means to ensure wetting by the continuous phase. A permanent surface modification is presented in the next chapter to solve this problem.

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Chapter 5

Surface Modification for Segmented Flow Microfluidic

This chapter describes a surface modification developed for microfluidic channel to ensure complete wetting by the continuous phase. PMMA channels are first coated with a silica nanoparticle layer rendering the surface superhydrophilic. Subsequent alkylation with chlorosilanes makes the surface hydrophobic allowing generation of aqueous droplet in hexadecane. Droplets containing bacterial culture were reliably generated without surfactant enabling us to test the resazurin assay in droplet format and make first comparisons with analog microplate experiments.

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5.1 Introduction

Droplet based microfluidic devices require complete wetting of the channel walls by the continuous phase to avoid pinning and smearing. However, most materials suitable for microfluidic devices require their surface to be modified to meet this requirement. Poly (methyl Methacrylate) (PMMA) has excellent transparency from UV to NIR wavelengths, low gas permeability and good dimensional stability. For these reasons, it was chosen as the optimal material for micro-droplet format of our real-time toxicity assay.

For segmented-flow microfluidics, a surfactant is generally added to the continuous phase and provides a dynamic coating that controls the wetting of the channel walls, but also prevents droplet coalescence [1]. For example, with the addition of oil or water soluble surfactants both, W/O and O/W emulsions in PMMA microfluidic chips can be prepared [2]. However, this comes at the cost of increased background fluorescence [3], leakage of small molecule through micelles [4], and microbial metabolism interferences [5]. Moreover, as surfactant concentration increases, merging droplets becomes difficult and irreproducible. In these situations, active measures are required (i.e. electro-coalescence [6]), which increases the complexity of the fabrication process and experimental setup. Therefore, if droplets do not come in contact with each other, a permanent modification of the channel surface is sufficient and more appropriate.

Several chemical approaches have also been proposed for the surface modification of PMMA. Aminolysis [7-9] and hydrolysis [10-11] to yield amine and carboxyl terminated surface respectively have been used for conjugating biomacromolecules. We initially tested hydrazine aminolysis [7] of PMMA for subsequent alkylation, but high surface density necessary to induce hydrophobicity generally requires more reactive reagents which tends to alter the surface topography and needs to be handled with care. Hydrophobic PMMA surface has been obtained by aminolysis with lithiated diamine and further derivatization with hydrocarbon chains [12]. Similar surface concentrations of amines has also been reported using ethylenediamine in DMSO but with loss of transparency due to solvent incompatibility, which is a major hurdle for PMMA modification [10]. Widely available

organosilane reagents have also been used on PMMA to introduce perfluorinated chains, amines and thiols after generating hydroxyls on the surface by oxygen plasma [13] or with strong reducing agent such as lithium aluminum hydride [14]. While this universal strategy is attractive, the resulting Si-O-C linkage on thermoplastic materials is susceptible to O-C hydrolysis. Covalent bonding to the surface is only temporary, and the coating should be treated as a physically adsorbed film, at best [15]. In the case of hydrophobic coating, hydrolysis is sterically hindered but stability is generally affected by defects in the self-assembled monolayer [16].

Wettability can also be enhanced by surface roughness [17] and super hydrophobic PMMA channels have been obtained by a plasma induced micro-roughness and C₄F₈ deposition [18]. We also attempted hydrophobic surface modification by atmospheric plasma polymerization of hexamethyldisiloxane (HMDSO). Although superhydrophobic coating could be obtained on PMMA slides, completely hydrophobic channels could not be made because of bonding issues or misalignment in case of patterned coating. Few methods to create superhydrophobic surfaces are applicable inside sealed microchannels. Direct superhydrophobic modifications have been reported for glass microchannel using titania nanoparticles [19] or aerogel coatings [20], but these are not applicable to plastics. Superhydrophobic surface are attractive for segmented flow microfluidic, but they remain elusive for disposable polymeric chip because of solvent and temperature limitations, need for optical clarity, control over coating thickness, adhesion to the substrate, etc.

To overcome these limitations, we test a hypothesis that silica nanoparticle (SiNP) can provide a primer layer to prepare anti-pinning surfaces for surfactant-free microfluidic devices. SiNPs are adsorbed from an acidic solution to form a near monolayer on the PMMA substrate. The PMMA/silica nanoparticle surface is superhydrophilic and subsequently silanized with alkylchlorosilanes to form a superhydrophobic surface. The effect of the SiNP diameter on surface morphology and subsequently droplet wetting is discussed. Finally, the effectiveness of the method is demonstrated with a surfactant-free microfluidic application where the kinetics of bacterial metabolism are monitored in real-time. The method was primarily developed for PMMA, however, preliminary experiments

on other thermoplastics suggest that this method is also applicable to a wider range of materials.

5.2 Experimental Methods

5.2.1 Materials and Chemicals

Hexadecane, 99%, HMDSO, 98%, iodododecane, 98% and n-Dodecyltrichlorosilane (DTS), 96% were purchased from Alfa Aesar, USA. Colloidal silica Ludox TM-50, Tetraethyl orthosilicate (TEOS), hydrazine hydrate, pentachlorophenol (PCP), resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) and brain heart infusion (BHI) broth were purchased from Sigma Aldrich, Singapore. Deionized water was used in all experiments. PMMA sheets 3 mm thick were obtained from Marga Cipta, Indonesia, 1 mm thick PVC slides from Art Friend, Singapore and 50 μm PET sheets from Professional plastics, Singapore.

5.2.2 PMMA Hydrophobic Surface Modification by Atmospheric Plasma Assisted Deposition of HMDSO

Hydrophobic coating was obtained by polymerization of HMDSO assisted by atmospheric plasma deposition (Plasmatreater AS400, Plasmatreat). Plasma parameters were optimized to prevent melting of the substrate and kept constant thereafter: nitrogen gas at 1700 L h⁻¹, voltage 70%, frequency 21 kHz, plasma cycle time 100%, jet velocity 30 m min⁻¹, raster offset 2 mm, nozzle distance 15 mm. HMDSO was sprayed with an airbrush (Badger 150) towards the substrate (orientation 1) or towards the top of the plasma jet (orientation 2) as shown in **Figure 5.1B-C**. The carrier gas was nitrogen at 1.25 bar and HMDSO was fed at 0.3 or 2 ml min⁻¹ with a syringe pump (Pump 11 Elite, Harvard Apparatus). Because HMDSO coating prevented thermal bonding, the coating was patterned by a lift-off process as shown in **Figure 5.1A**. The coating was 200 μm wider than the channel to allow manual alignment and both, the channel and cover plate were coated.

5.2.3 PMMA Surface Modification through Aminolysis

Aminolysis of PMMA slides was done in 1:1 (v/v) mixture of hydrazine hydrate and methanol at 50°C for 48h. Alkylation was tried in 1:10 (v/v) iodododecane in hexane for 36h or in aqueous solution containing 2 mM iodododecane, 2mM NaHCO₃ and 5 mg of surfactant Sodium dodecyl sulfate (SDS).

5.2.4 Synthesis and Characterization of Silica sols

Commercially available SiNPs Ludox TM-50 were diluted to 0.5 wt% in water, and the pH was rapidly adjusted to pH 2 with HCl; this sol was used in most experiments. To study the size effect, nanoparticles were also synthesized from TEOS as summarized in **Table 5-1**. Smaller particles were synthesized using an emulsion method where the base was replaced by NaOH [21]. TEOS was added dropwise to 50 mL of 1mM NaOH while stirring. The solution was stirred for 24h and the pH was rapidly reduced to 2 with HCl. Larger particles were synthesized from the Stöber method with varying ethanol/water ratios [22]. Ethanol was mixed with 1.5 mL TEOS and water premixed with 3 mL 25% ammonia was added at once. Solution was stirred at room temperature for 2 hours. Finally, 2 mL of the sol was rapidly mixed with 18 mL of 1M HCl. Particle size was measured by dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, Malvern Instruments, UK).

Table 5-1 Summary of nanoparticles used to study the effect of size on surface modification

Method	Synthesis conditions	Z-average (nm)	PDI
commercially available TM-50	-	35	0.10
emulsion	1 mL TEOS	9	0.28
emulsion	5 mL TEOS	15	0.14
Stöber	35mL EtOH, 18 mL H ₂ O	169	0.05
Stöber	30mL EtOH, 23 mL H ₂ O	97	0.04
Stöber	25mL EtOH, 28 mL H ₂ O	39	0.30

5.2.5 PMMA Hydrophilic and Hydrophobic Surface Modification Based on Silica Nanoparticles

The PMMA surface was modified according to the scheme depicted in **Figure 5.3**. PMMA slides cut to 1.5 cm x 6 cm were sequentially washed with ethanol and water and blow dried with a stream of compressed air. SiNPs were first adsorbed on the PMMA surface by dipping the slides in silica sol and the slides were rinsed with water and dried in an oven at 90 °C for 15 minutes; this cycle was repeated up to four times. After the last cycle, the drying time was extended to one hour. Subsequently the surface was silanized by immersion for one hour in 0.5% (v/v) DTS in hexane previously dried over magnesium sulfate. Finally, the slides were sequentially rinsed with hexane and water and kept in a desiccator until characterization. PET and PVC were coated in similar fashion except the drying temperature was reduced to 70 °C because of their lower glass transition temperatures.

Contact angles (CAs) were measured with a goniometer (OCA 15Pro, DataPhysics, Germany) using the tilt-plate method with 20 μL drop volume and 0.5 $^{\circ}\text{s}^{-1}$ tilt rate. Advancing/receding CAs were taken right before the drop started moving, defined as the point where the drop base diameter is the largest (**Figure 3.7**). For water in oil CAs the substrate was immersed in hexadecane, and a 6 μL water drop was deposited on the surface and allowed to equilibrate for one minute before measurement. Standard deviation of 4 drops per substrate is reported. Samples after SiNPs adsorption were imaged by scanning electron microscopy (SEM) (JSM-6340F, JEOL, Japan) with a thin platinum coat sputtered under argon at 10 mA for 120 s (JFC-1600, JEOL, Japan); the contrast of the SEM images was enhanced by histogram equalization. RMS surface roughness was characterized by atomic force microscopy (AFM) (Cypher S, Asylum Research, USA) in attractive mode (i.e. non-contact) with a 160-kHz tip (NCSTR, Nanoworld, Switzerland). The scan area was 1 μm x 1 μm , and scan rate 0.8 Hz; the values reported are the average of 4 scans at different locations. Transmission spectra were acquired with a UV-VIS spectrophotometer (Varian Cary 50 UV-Vis). Baseline was acquired without any sample and transmission of 3mm thick PMMA sheets were measured with 90° incident light.

5.2.6 PMMA Microfluidic Chip Fabrication and Hydrophobization

PMMA microfluidic chips were fabricated deep UV etching of PMMA slides as described in **section 4.2**. The channels were coated in similar fashion as for the flat substrates. One mL of each solution was manually flushed in sequence at a high flow rate ($\sim 2 \text{ ml min}^{-1}$) to remove trapped air bubbles. The excess water was removed by blowing compressed air. Prior to DTS silanization the quality of the silica layer was checked by generating air plugs in a water filled channel. The silica coating cycle was repeated until pinning of air plugs under static conditions was eliminated (typically 3-4 times). Micrograph images of the channel at different stages were taken with a bright-field microscope (BX53, Olympus) equipped with a CMOS camera (Infinity1, Lumenera).

5.2.7 Microfluidic Device Operation

The setup with camera, LEDs and syringe pumps described in **section 4.2** was used. Capillary number was estimated from the average droplet velocity in a straight section of the incubation channel:

$$C_a = \frac{\mu U}{\gamma} \quad (\text{eq. 5.1})$$

where μ is the viscosity of the continuous phases, U the droplet velocity and γ the interfacial tension between the 2 phases [23]. The shear stress at the wall τ_w in the centre of the channel is estimated from the Purday approximation [24] for rectangular channel:

$$\tau_w = \frac{2\mu Q}{wh^2} \left(\frac{m+1}{m} \right) (n+1) = 310 \frac{\text{dyn}}{\text{cm}^2} \quad (\text{eq. 5.2})$$

Where μ is the viscosity, Q the flow rate and $m = 1.7 + 0.5(h/w)^{-1/4}$, $n = 2$ for $h/w > 1/3$ are empirical parameters with the main channel width $w=800 \text{ }\mu\text{m}$ and height $h=100 \text{ }\mu\text{m}$.

5.2.8 *E. faecalis* Bacterial Viability Experiments with Cu^{2+} , Ni^{2+} and PCP

Enterococcus faecalis, a facultative strain commonly found in anaerobic digesters was used for this experiment [25]. *E. faecalis* OG1RF (ATCC® 47077™) (100 μL) was injected into 10 mL BHI sterile medium previously bubbled with N_2 . The culture was incubated overnight (stationary phase) and was stored at 4 °C until use (<24 h). Final bacterial density inside the droplets was estimated at $3.7 \times 10^7 \text{ cell mL}^{-1}$ by the acridine orange direct count method [26].

Droplets containing toxicants mixed with bacteria and resazurin were generated on the chip. Stock solutions for the four aqueous inlets were 1) ddH₂O, 2) Toxicants (NiCl_2 or CuCl_2 166.6 mg L^{-1} in ddH₂O or PCP 166.6 mg L^{-1} in PBS), 3) *E. faecalis* ($\text{OD}_{600} \approx 0.5$) and 4) resazurin dissolved in PBS (125 mg L^{-1}). Resazurin and *E. faecalis* were injected at 0.2 $\mu\text{L min}^{-1}$ while the toxicants and PBS flow rates were adjusted to give a total aqueous flow rate of 1 $\mu\text{L min}^{-1}$. To create different toxicant concentrations, the flow rate ratio was automatically changed every 10 droplets, and the first 3 droplets after each transition were discarded resulting in 7 replicates. The kinetics of each sample were fitted with a sequential pseudo-first order model obtained under the assumption that the reactions were irreversible under anaerobic conditions per **Equation 3.1**. A one-way ANOVA test with post-hoc Tuckey HSD was used to compare the fitted parameters.

5.3 Results and Discussion

The aims of this work were to develop a surface modification procedure for PMMA microchips that 1) ensure complete wetting by the continuous phase in segmented-flow applications, 2) avoid the use of surfactants, 3) maintain optical transparency, 4) is durable with respect to shear stress and fouling.

5.3.1 Hydrophobic Modification of PMMA Prevents Bonding

Plasma enhanced chemical deposition is an attractive method because it allows polymerization of otherwise non-reactive monomers and simultaneous activation of the

surface, thus potentially creating covalent bond to any surface. HMDSO deposition was attempted on PMMA with an atmospheric plasma jet described in details elsewhere [27]. HMDSO coating can vary from glass-like to PDMS-like as it is fed lower in the plasma jet [28]. To try retain the monomer structure as much as possible and create a PDMS-like hydrophobic layer, HMDSO was sprayed with an air brush below the nozzle, either towards the jet or the substrate. When the spray was oriented towards the substrate, transparent coatings were obtained with a CA of 81° at most but decreased CA hysteresis compared to pristine PMMA as shown in **Figure 5.1**. This CA is significantly lower than 102° previously reported and is likely due to incorporation of oxygen in our open environment [29]. When the spray was oriented towards the jet, particles were formed and, at high monomer rate, the film was slightly white and exhibited superhydrophobic properties with an advancing CA of 162° and 5° hysteresis. As visible in SEM images (**Figure 5.1F-G**) superhydrophobicity originates from dendritic structures resulting in a Cassie wetting regime. As often observed with superhydrophobic surface, the coating was mechanically weak and could be removed by a finger touch but was resistant to solvent wash (water, hexane, hexadecane) thus making it suitable for our application.

Figure 5.1 Hydrophobic surface modification by atmospheric plasma assisted deposition of HMDSO. (A) Lift-off process to coat the channel. (B) HMDSO spray and plasma jet oriented towards the substrate. (C) HMDSO spray oriented towards the jet, giving opportunity for reaction to start before reaching the substrate. (D) Dynamic CAs as function of HMDSO spray orientation and feed rate. (E) Water in hexadecane droplets in HMDSO treated channel (cover plate and channel, orientation 2, 2 mL min⁻¹). Misalignment during bonding results in some untreated edges. (F-G) SEM images of superhydrophobic plasma polymerized HMDSO film at low and high magnification (orientation 2, 2 mL min⁻¹).

HMDSO coating prevented thermal bonding of PMMA sheets and had to be patterned to coat only the channels area according to the lift-off process depicted in **Figure 5.1A**. This

process was however poorly repeatable and good quality chips could not be obtained. When the coating was the same as the channel width, alignment was impossible as shown in **Figure 5.1E**. The coating was made wider to simplify alignment but the bond strength (if bonded at all) was too low and chips delaminated during handling. The HMDSO coating was also sometimes partially removed while stripping the photoresist with ethanol, especially on the etched side of the channel because of its higher solubility compared to pristine PMMA. Finally, when filled with oil HMDSO coating matches the refractive index of hexadecane but was too rough to be used with fluorocarbons (channel appearing white on camera).

5.3.2 Surface Alkylation Requires High Grafting Density to Create Hydrophobic Surface

To avoid bonding issues, a first wet chemistry method depicted in **Figure 5.2** was attempted to modify enclosed channels. First amines were introduced by hydrazine aminolysis of the methyl ester groups of the PMMA surface [7]. The contact angle was reduced from 70° on pristine PMMA to 52°, indicating at least partial aminolysis. Subsequently alkylation was attempted with hexane solution of iodododecane but the contact angle only increased to 63°, still lower than that of pristine PMMA. It was thought that poor solubility of the by-product hydrogen iodide in hexane might limit the reaction. Therefore, the reaction was also attempted in aqueous solution based on reported protocols for alkylation of amines in solution [30], but without better success.

Figure 5.2 Attempted PMMA hydrophobic surface modification scheme B: hydrazine aminolysis followed by alkylation with iodododecane. CAs corresponding to each step are indicated above.

A major limitation of PMMA is its incompatibility with majority of polar aprotic solvents that would be ideal for Sn2 reaction. Another factor to consider is surface coverage. For comparison, self-assembled monolayer of alkyltrichlorosilanes on silicon wafer have ~ 4.5 -5 groups nm^{-2} resulting in contact angle of ~ 110 - 115° [31]. Soper et al. reported similar modification scheme, albeit pyrophoric reagents were used for aminolysis step [12]. They obtained higher contact angle ($103 \pm 10^\circ$) with subsequent octadecane-1-isocyanate alkylation but even then, unreacted amines remained accessible to water. Although dynamic contact angles were not reported, high contact angle hysteresis is to be expected from chemically heterogeneous surface, which would impede droplet mobility [32]. Given the likelihood that even if both steps were optimized it might not eliminate pinning sites, this approach was abandoned.

5.3.3 Silica Nanoparticles near Their Isoelectric Point Adsorbs on PMMA

Trichloroalkyl silanes were considered instead monofunctional alkylation reagents because they laterally crosslink, forming a densely-packed monolayer with potentially more molecules than reactive groups present on the surface [31]. It however requires the presence of hydroxyls on the surface. As mentioned in introduction, hydroxyls can be

introduced on PMMA by plasma or strong reducing agents but it would results in Si-O-C linkage that is poorly stable towards hydrolysis [15]. Instead we introduced hydroxyls with an intermediate SiNPs layer, with the added benefit of providing control over surface roughness. Coating of complex 3D substrates can be obtained by deposition of charged nanoparticles alternated with a polyion of the opposite charge using a layer-by-layer (LbL) deposition technique where surface roughness and coating thickness is controlled by the number of deposition cycles. SiNP LbL based coatings have been reported for both extreme hydrophilicity [33] and hydrophobicity [34-35]. More specific to microfluidic applications, Lee et al. developed a spray LbL technique to create a hydrophilic surface on a cyclic olefin copolymer [36]. Their method still requires O₂ plasma pretreatment, and regions outside the channel have to be masked to allow for bonding. Superhydrophobic surfaces are generally created by removing the polycation by calcination to leave a neat silica layer whose surface chemistry can easily be modified; however, calcination is not applicable to thermoplastic substrates. As an alternative to the polycation approach, we relied on the unusual stability of silica sols at their isoelectric point due to the slow condensation of silanol groups to adsorb a dense SiNP layer on the substrate. As illustrated in **Figure 5.3A**, PMMA was first coated with SiNPs which increases the surface roughness and act as a primer for further chemical modification. In a second step alkyl chains were attached to the silica surface through silane chemistry to render the surface hydrophobic.

Figure 5.3 (A) PMMA hydrophobic surface modification scheme. The surface is first coated with silica nanoparticles which render the surface superhydrophilic. Complete surface coverage is obtained with 1-3 cycles depending on particle size. In a second step, the silica layer is rendered hydrophobic by chemical modification with DTS. (B-C) Optical micrographs showing transition from, (B) partial wetting before modification to, (C) complete wetting by the continuous phase after hydrophobic modification.

5.3.4 Complete Coverage of the PMMA Surface Is Reached After 3 Deposition Cycles of Silica Nanoparticles

SiNPs were first deposited on PMMA slides resulting in a more hydrophilic surface. The Z-average size of the nanoparticles measured by DLS was 35nm with a polydispersity index of 0.1, and remained unchanged throughout the coating procedure. As shown in **Figure 5.4A**, the static CA decreases with the number of silica coating cycles, and complete wetting is reached after 3 cycles. Complete residual wetting by the retreating drop was visually observed (i.e. 0° receding angle) already after one silica coating cycle. Without heating the substrate, SiNPs were easily removed by basic solutions (e.g. 0.1 mM NaOH) and the CA of pristine PMMA was recovered. A drying temperature of 90°C was chosen as a compromise to maximize SiNPs adhesion, while maintaining the channel integrity, when applied to the microchip. Heating promotes covalent crosslinking of the SiNPs via silanol condensation thus creating clusters that cannot be redispersed [37]. Heating can also provide better adhesion to the substrate due to particle sink. Nanoparticles engulfment in polymer matrix by capillary rise infiltration above the T_g has been reported [38], and it has also been observed for SiNP on PMMA below T_g ($\sim 110^\circ\text{C}$), albeit at a slower rate [39], which means channel deformation can be easily prevented.

Figure 5.4 (A) Water contact angle and corresponding surface roughness after repeated deposition cycles of SiNP on PMMA slides. (*) Contact angles below 5°. (B-D) Air bubbles generated in water-filled PMMA microchannel: (B) untreated, (C) after 1 SiNP coating cycle; air bubble remains pinned on the side wall, (D) after 6 SiNP coating cycles. Repeated SiNP deposition cycles reduce defects thus preventing bubble pinning.

Surface topography of SiNP coated PMMA was characterized by SEM and AFM. After the first coating cycle, SiNPs partially covered the PMMA surface and formed island-like clusters, as can be seen in **Figure 5.5A**. Subsequent coating cycles fill the remaining gaps with full coverage reached after 3 cycles. AFM micrographs of silica coated PMMA confirms the full surface coverage after 3 cycles, with the underlying PMMA surface no longer detectable by either method, as seen in **Figure 5.5F** (inset). Surface roughness increased to a RMS roughness of 8 nm after the first cycle compared to 1.4 nm on pristine PMMA, and did not change significantly with subsequent cycles as shown in **Figure 5.4A**.

Wettability is dependent upon surface chemistry and roughness. Unlike in the traditional LbL approach where the surface roughness gradually increases with the number of bilayers [40], the surface roughness (8 nm) was independent on the number of cycles. SEM and AFM analysis after repetitive coating cycles suggest that the decrease of CA is due to an increase in surface coverage rather than a shift in surface roughness. This gap-filling effect is possible because of the lack of electrostatic repulsion of the silica sol near its isoelectric

point. Repeated coating cycles effectively make the surface more chemically homogenous. This is important as chemical heterogeneities will pass on to the next silanization step and cause unwanted CA hysteresis [41].

Figure 5.5 (A-C) SEM images of PMMA slides after 1, 2 and 3 cycles of 35 nm SiNP coating. Gaps between Island-like clusters are progressively filled with repeated SiNP deposition cycles. (D-F) Corresponding AFM images, 32 ± 6 nm particle height was estimated from local maxima on images with visible reference substrate (i.e. after 1 cycle). Inset of panel (F) is the same AFM image after local contrast enhancement highlighting the presence of nanoparticles in the low height regions.

While helpful in controlling wetting, too much roughness can also scatter light and impede optical measurements. As shown on **Figure 5.6D**, pristine PMMA transmit 92% of the light in the visible region while most of the light not transmitted is reflected at the air-PMMA interface [42]. The roughness resulting from SiNPs slightly improves transmission in the lower wavelengths region because the nano-porous coating reduces reflection at the air interface [43].

Figure 5.6 (A-C) Dynamic contact angles after SiNP coating and subsequent DTS silanization as function of: (A) SiNP coating cycles with 35 nm SiNP, (B) nanoparticles size with 3 coating cycles, (C) substrate material with 3 coating cycles. (D) UV-Vis transmission of 3 mm thick PMMA sheets. The silica nano-coating is transparent and even slightly increases the transmission compared to pristine PMMA by reducing reflections at the air interface. Further DTS silanization does not affect optical performances.

5.3.5 Hydrophobic silanization of silica coated PMMA prevents pinning of water droplet in hexadecane

The deposited SiNPs were hydrophobically modified with DTS, an alkylsilane that forms self-assembled monolayers and reacts with silanol groups to form covalent bonds with the underlying silica layer [44]. The subsequent change in surface wetting was monitored by dynamic CA measurements in air. As seen in **Figure 5.6A**, a maximum advancing angle

close to 140° is reached with one SiNP cycle. Two additional SiNP cycles are required to reach a stable receding angle of $\sim 84^\circ$. Contrasting with this large CA hysteresis (56°), when air was replaced by hexadecane, advancing and receding CAs on PMMA slides coated with 3 SiNP cycles and DTS silanization were $165 \pm 2^\circ$ and $161 \pm 4^\circ$, respectively.

High CA and large CA hysteresis are characteristics of the Wenzel regime on intrinsically hydrophobic and rough surfaces ($CA > 90^\circ$). Water penetrates in the nano-texture, resulting in strong adhesion, sometimes referred as petal effect. It is undesirable for segmented flow microfluidics as it will result in poor droplet mobility (i.e. pinning). On the other hand, Cassie regime is characterized by low CA hysteresis due to air pockets trapped in the coating and is energetically favored when the surface roughness is above a critical threshold [45]:

$$r_f > \frac{\cos \theta_C}{\cos \theta_Y} \quad (\text{eq. 5.3})$$

where r_f is the roughness defined as the ratio of surface in contact with the liquid to its projected area, θ_Y is the Young CA and θ_C the apparent CA in Cassie state. In fact, SiNP based transparent superhydrophobic coatings with low CA hysteresis have been obtained with surface roughness as low as ~ 50 nm [34] and ~ 60 nm [35], whereas in our case the surface roughness was 8 nm. **Equation 5.3** also indicates that the minimum roughness required is reduced when the Young contact angle increases which is the case when air is replaced by oil; the Young CA on alkylated surface changes from 104.0° for water in air to 163.6° for water in hexadecane [46]. Hexadecane, effectively requires less stringent conditions than air to favor the Cassie regime [47]. This hypothesis is supported by the observation that CA hysteresis was reduced from 56° to 4.1° when air was replaced by hexadecane. Intermediate mixed wetting states are also possible and while increasing the roughness would favor low adhesion Cassie wetting regimes, it must be balanced with loss of transparency due to light scattering as discussed in the next section.

5.3.6 Optimal hydrophobic coating is obtained with 35 nm Silica nanoparticles

SiNP deposition cycles followed by DTS silanization were applied to PMMA slides using different nanoparticle sizes. Complete surface coverage was reached after only one deposition cycle for 9 nm SiNPs (**Figure 5.7**) versus three cycles required for 35 nm SiNPs (**Figure 5.5D-F**). As shown in **Figure 5.6B**, the advancing CAs for the smallest 9 nm SiNPs were similar to that of a flat silicon wafer silanized with DTS [48]. These particles were also tested for coating on the microfluidic channels and allowed stable operations for a few hours after which macroscopic cracks leading to droplet pinning appeared (not observed on flat PMMA substrates). Adsorption of 97 and 169 nm SiNPs was insufficient to retain a complete wetting film, and solid residues were visible to the naked eye after drying. Advancing and receding fronts were moving in a stepwise fashion resulting in the larger standard deviations reported. Hence, these large SiNPs were deemed unsuitable and were not investigated further.

Figure 5.7 AFM images of PMMA slides: (A-C) after 1, 2 and 3 cycles of 9 nm SiNP coating. Complete surface coverage is already obtained after 1 cycle (compared to ~ 3 cycles for 35 nm SiNP), (D) pristine PMMA. Reported roughness are the average of 4 scans at different locations.

Overall the trend observed is that the smaller the particles, the better the coverage is after a single cycle. From this aspect, smaller (9 nm) particles would be advantageous but cracks formation precludes their use for long term operations. It has previously been reported that coatings manufactured from larger particles (50 nm) are less prone to cracking than those from small ones (7 nm) [43]. Particles larger than 100 nm cannot be used with our method due to poor adsorption and non-uniform coating. SiNPs between 35 nm and 100 nm could

result in the critical surface roughness of 20 to 60 nm that leads to Cassie wetting regime in air while maintaining optical transparency [34-35]. It is however expected to require more coating cycles and is not necessary when air is replaced by oil as stated above. In conclusion, 35 nm SiNPs offer the best compromise between surface roughness, surface coverage and transparency.

5.3.7 Surface Modification with SiNPs Also Applies to PET and PVC

Figure 5.8 AFM images of SiNP coating method applied on PET and PVC substrates: (A) pristine PET, (B) PET coated with 3 cycles of SiNPs, (C) pristine PVC, (D) PVC coated with 3 cycles of SiNPs.

Because our method does not rely on chemistry specific to PMMA, it is also applicable to PVC and PET. Similar to PMMA, complete surface coverage is obtained after 3 SiNPs deposition cycles on both PET and PVC as illustrated in **Figure 5.8**. CAs before and after modification are shown in **Figure 5.6C**. After modification, only the receding contact

angles between PET and PVC were slightly different ($p=0.029$) thus demonstrating potential of our method for other plastic substrates and potentially a wider range of polymeric substrates. Silane chemistry has a long history for surface modification of inorganic substrate such as glass but is inherently unstable when use directly on organic substrates due to the poor hydrolytic stability of the resulting Si-O-C linkage [15]. Thus the SiNPs layer could also act as a primer layer for silane reagents other than DTS and offer an alternative to plasma or chemical activation of organic substrates.

5.3.8 Heat Treatment of SiNP Coated UVC Etched PMMA Induces Nano-Wrinkles

Our microchannels are composed of 3 UVC etched sides and a pristine PMMA cover. To quantitatively evaluate the impact of UVC etching on the final hydrophobic coating, a slide was etched and coated with 3 SiNP cycles followed by DTS silanization. The advancing angle did not change significantly ($p=0.501$) while the receding angle was 5° higher ($p=0.017$). This higher CA can be explained by a 7.4 nm increase in surface roughness on etched PMMA because of the formation of nano-wrinkles. As shown in **Figure 5.9A-B**, the surface roughness increases immediately after etching but drops back down after heating to 110°C (i.e. during thermal bonding). However, after the SiNP deposition and drying at 90°C , worm-like wrinkles were formed resulting in overall increased surface roughness from 8 to 15 nm. Similar patterns to the one shown in **Figure 5.9C** were observed on all scanned areas while none were found when the coating was applied to pristine PMMA.

PMMA surface exposed to UV (254 nm) undergoes chain scission resulting in lower molecular weight and lower glass transition temperature of the top surface [49]. It also increases the dissolution rate in organic solvents (e.g. isopropanol) thus allowing selective removal of the exposed areas [50]. Because there is a gradient of molecular weight rather than a well-defined transition, the layer near the etched surface is likely to have a lower glass transition than the bulk. This translates to a higher thermal expansion coefficient and wrinkles formation commonly observed when thin layer having a high modulus are coated on substrate with much lower modulus. Similar behavior with a strong dependency on the

Tg has previously been reported for heat treated Au film on top of PMMA [51]. Since the resulting surface roughness is still well below the diffraction limit and does not reduce transparency, this effect is beneficial for superhydrophobic surfaces with low CA hysteresis but not absolutely required for water in hexadecane and positively impacts performances if at all.

Figure 5.9 AFM images of SiNP coating on etched PMMA instead of pristine: (A) etched PMMA. Etching increases the surface roughness to $3.4 \pm 0.8 \text{ nm}$ compared to $1.4 \pm 0.7 \text{ nm}$ on pristine PMMA. (B) Etched and annealed PMMA. Annealing etched samples at 110°C smoothen the surface. (C) Etched and annealed PMMA coated with 3 cycles of SiNPs, wrinkles are formed. (D) PMMA coated with 3 cycles of SiNPs, scan range extended to $3 \mu\text{m}$ for comparison with left image, no wrinkles are formed. Reported roughness are the average of 4 scans at different locations.

5.3.9 Reproducibility of Droplet Generation Is Impeded by Coating Defects Only at Low Flow Rates

Figure 5.10 Wetting regimes in uncoated and coated PMMA channels, fluorescent dye was added to aqueous phase. (A) Partial wetting in uncoated channel, aqueous plugs come in direct contact with the walls. (B) Oil droplets in water inside silica coated channel. (C) Water droplets in oil inside channel silanized with DTS. (D) In uncoated channel, addition of bacterial culture results in fouling of the walls and erratic flow. (E) With DTS coating, water droplets in oil are unaffected by bacterial culture.

The modification procedure was also applied to microchannels. After the initial SiNPs deposition, air bubbles were introduced into water filled channels to qualitatively assess the formation of pinned bubbles. A change from partial wetting on pristine PMMA to complete wetting by the water phase was observed after the first coating cycle, as illustrated in **Figure 5.4B-D**. However, when the bubble velocity approached static conditions pinning of the air bubbles was observed. The number of pinned bubbles was qualitatively reduced as the number of silica coating cycles was increased. SiNP coated PMMA also allowed generation of hexadecane droplets in water continuous phase (**Figure 5.10B**). The

coating could withstand high flow rate corresponding to shear stress of at least 310 dyn cm^{-2} .

With subsequent hydrophobic silanization, a similar change from partial wetting by the oil phase on pristine PMMA to complete wetting was also observed for water in hexadecane filled channels, as shown in **Figure 5.3B-C** and **Figure 5.10A-C**. Under dynamic flow conditions in a microchannel, a lubricating oil film is present between the droplet and the wall due to the balance between capillary forces and viscous entrainment. When the flow rate approaches stationary conditions, the lubricating film gets thinner, and below a critical capillary number intermolecular forces dominate [52]. If the channel surface has more affinity for the continuous phase, the film reaches an asymptotic low thickness. In the opposite case, the lubrication film collapses and the channel surface is wetted by the dispersed phase [53]. In our experiments, we noticed this transition when using less than 3-4 SiNP coating cycles and DTS silanization. Droplet pinning was observed when the droplet velocity was below 5 cm min^{-1} ($Ca \approx 5.6E-5$, corresponding to a total flow rate of $4 \mu\text{l min}^{-1}$ for our channel geometry). Above it we had no problem generating stable droplets with only two SiNP deposition cycles, because the dispersed phase did not contact the walls. We attributed this pinning to the presence of defects in the coating as it was qualitatively reduced by increasing the number of coating cycles. This analysis correlated for air bubbles in a hydrophilic channel filled with water. Therefore, air bubble pinning is a qualitative indicator for coating defects, and was used to determine when the silica layer is optimal and DTS silanization should be applied.

5.3.10 Bacteria Droplets Can Be generated Without Surfactant

As stated before, the heating step in the coating process prevented removal of SiNPs by 0.1 mM NaOH . Since mildly basic solutions are not uncommon for biological assays and other applications of segmented-flow microfluidic, stability under dynamic flow conditions was further confirmed. Droplets of 0.1 mM NaOH were generated at $15 \mu\text{l min}^{-1}$ with intermittent increase to 2 mL min^{-1} with no degradation of the coating observed as shown in **Figure 5.11**. Practical application also involving bacterial cultures. *E. faecalis* injected

into untreated PMMA microchannel filled with hexadecane (partial wetting regime) resulted in droplet pinning and uncontrollable droplet generation within a few minutes as illustrated in **Figure 5.10D**. Microchannels coated with SiNPs and DTS silanization were challenged under the same and the same complete wetting regime as with distilled water was retained, even with droplets containing a concentrated bacterial culture as shown in **Figure 5.10E**. The same chip was used for several experiments and no degradation (i.e. droplet pinning) of the coating was observed after storage for at least 6 months.

Figure 5.11 The stability of the coating was tested with 0.1 mM NaOH (pH 10) as the aqueous phase, no degradation was observed for at least 48 hours for both coatings: (A-C) hydrophilic SiNP (oil in water) and (D-F) hydrophobic SiNP+DTS (water in oil).

Avoiding surfactants when possible can minimize the extraction of compounds into the oil phase and limit exchange between droplets, thus adhering more closely to the idealized picture of isolated droplet microreactors [54-55]. Furthermore, it also allows sequential injection of reagents into existing droplets without resorting to using active methods such as electrocoalescence. In addition to characterization with distilled water, our surface modification approach is also effective in a more demanding, real application using a bacterial culture which contains surface fouling proteins and other secreted surface active molecules. We also anticipate that the coating nano-rough features will extend long-term

resistance to fouling compared to flat hydrophobic surfaces due to continuously renewed oil pockets being trapped in the coating [56].

5.3.11 Viability of Facultative Bacteria in Micro-Droplets Can Be Analyzed Within 5 Minutes

Figure 5.12 Resazurin reduction by *E. faecalis* spiked with different (A-B) CuCl_2 and (C-D) NiCl_2 concentrations. (A-C) Fluorescence kinetics: Resazurin is reduced to fluorescent product resorufin by bacteria. When bacteria metabolism is inhibited by the presence of metal, the resazurin conversion rate is reduced. Each curve is the average from 7 droplets. Dotted lines represent fitting from **Equation 3.1**. (B-D) Fitted rate constants K_1 and K_2 for a sequential pseudo-first order model as function of metal concentration.

The microfluidic chip depicted in **Figure 4.1** was hydrophobized and used to monitor real-time metabolism of *E. faecalis* through the reduction of resazurin to its fluorescent product

resorufin. Droplets with CuCl_2 and NiCl_2 concentration ranging from 0 to 100 mg L^{-1} were independently generated at the first T-junction, and quickly homogenized in the meandering channel due to internal recirculating flow [23]. *E. faecalis* was then injected into existing droplets and flowed through the next wavy section of the channel (pre-incubation) for approximately 1 min before resazurin was injected. The fluorescence intensity of each droplet was then measured in real-time while flowing along the channel according to the procedure detailed in **Chapter 4**.

Figure 5.12 shows the average kinetic curves ($n=7$) of resazurin reduction in the presence of different nickel concentrations. Negative values on time-axis correspond to pre-incubation of bacteria with heavy metal. Resazurin was added at $t=0 \text{ s}$, and it took approximately 15 s to homogenize the droplets. In all cases the resazurin reduction rate was not linear, but decreased with time following an irreversible sequential pseudo-first order kinetics. Fitting of individual curves from $t=15 \text{ s}$ (i.e. after mixing resazurin) have an R^2 values greater than 0.99, and the average fitted rate constants for each concentration are shown in **Figure 5.12**. Heavy metals alone (i.e. without bacteria) had no effect on the reduction of resazurin, as measured within the timescale of the experiment. However, the resazurin reduction rate by *E. faecalis* was significantly reduced in the presence of 10 mg L^{-1} CuCl_2 or 30 mg L^{-1} NiCl_2 and higher concentrations compared to the positive control. The rate K_2 for the second conversion to dihydroresorufin increased with addition heavy metals. It might however be an artefact due to the short timescale recorded (no decrease in fluorescence observed) or that the assumption for a pseudo first order model does not hold ($[A]_0 \ll K_m$, c.f. **Section 3.2.3**), which is addressed in **Chapter 6**.

5.3.12 The Resazurin Assay Is More Sensitive and Faster in Microdroplets Than 96-Well Plates for Heavy Metals but Not for PCP

Toxicity results were also compared with analog experiments on 96-well plates [57]. **Figure 5.13** shows the resazurin reduction kinetics by *E. faecalis* with 50 mg L^{-1} Cu^{2+} compared to control. A plateau was reached in about 15 min compared to less than 5 minutes in droplets. The reduction rate in the first minutes dropped by approximately 10%

with the addition of copper whereas in droplets almost 50% inhibition was observed (**Figure 5.12**). Droplets are continuously mixed as they flow along the channel [23] but in microplate where shaking is intermittent, resazurin reduction could be limited by diffusion. Inner filter effects at high resazurin concentration and longer optical path in microplate (1 cm vs 100 μm) can also reduce the intensity measured in the first minutes. In microfluidic format, incubation time is limited to about 5 minutes but in microplate, kinetics was recorded for an hour. At 30 minutes, the fluorescence started to decrease in control, indicating secondary conversion to dihydroresorufin, which was not observed with copper. An assay based on the second reaction (i.e. using resorufin instead of resazurin) might be more sensitive but two factors need to be considered. First, greater sensitivity can also be due to longer exposure to copper. Second, the resorufin reduction rate is slower which would result in longer detection time and might not be achievable in microfluidic format.

Figure 5.13 Resazurin reduction by *E. faecalis* spiked with copper, measured in microplate by Dr. Chen Jianlin. Inset shows magnification of the first 10 minutes.

On the other hand, the presence of the organic toxicant pentachlorophenol (PCP) was only detected above 70 mg L^{-1} as shown in **Figure 5.14**. In comparison, on microplate, 5 mg L^{-1} PCP and above resulted in significant inhibition after 4 minutes [57]. In microfluidic format, the concentration of hydrophobic compounds in droplets is reduced because of partitioning in the hexadecane continuous phase. This problem is addressed in **Chapter 7**.

Figure 5.14 Resazurin reduction by *E. faecalis* spiked with different PCP concentrations in droplet format with hexadecane as continuous phase.

5.4 Conclusion

In this work, we have reported on a method to coat PMMA channels and other thermoplastics for the formation of O/W and W/O droplets, both without surfactant. Hydrophilic surface was first obtained by deposition of a neat silica nanoparticle layer inside PMMA microchannel which is unachievable by conventional coating methods. Subsequent silanization with DTS render the surface superhydrophobic, ensuring complete wetting by the continuous oil phase without compromising the optical properties of PMMA. No degradation was observed over 6 months, and we demonstrated long term surfactant free operation with aqueous droplets containing high concentrations of adsorption prone

bacteria. Our method is also effective on PET and PVC and will likely apply to other thermoplastics. Surface modification of plastics often requires lengthy development of material specific protocols. We envision that this silica layer can serve as a primer to leverage the vast silane chemistry readily available.

Finally, we used this method to transfer the resazurin viability assay onto a microfluidic chip (from a 96-well plate) to develop a continuous toxicity monitoring test for anaerobic digestion. In its current configuration, the microfluidic platform is limited to detection of hydrophilic compounds albeit the resazurin kinetics differed significantly from microplates. A more detailed analysis of the resazurin kinetics with respect to starting concentration, bacterial density, etc. is presented in **Chapter 6** while the detection of hydrophobic compounds such as PCP is addressed in **Chapter 7**.

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Chapter 6

Resazurin Reduction Kinetics in Segmented-Flow Microfluidic

*This chapter investigates the details of the resazurin assay in microfluidic format. Continuous mixing effect on the resazurin reduction rate and its impact on apparent toxicity are discussed. A method to generate droplets serial dilution is introduced and used to assess the resazurin kinetics as function of substrate concentration, bacterial density and heavy metals inhibition. Resazurin kinetics and copper inhibition are also studied as a function of bacterial growth. Finally, the effect of five heavy metals, Hg^{2+} , Cu^{2+} , Co^{2+} , Zn^{2+} and Ni^{2+} on bacteria *E. faecalis* and yeast *Saccharomyces cerevisiae* are compared.*

6.1 Introduction

Fluorescence signal from resazurin conversion can be detected within minutes, however manufacturer instructions and majority of reports in literature recommend pre-incubation with toxicants and incubation with resazurin for at least an hour each [1-2]. Relatively low bacterial densities (10^5 - 10^6 cell mL⁻¹) in log phase are also suggested. All of which are either impractical or simply impossible to fulfil for rapid and continuous toxicant detection. Segmented-flow microfluidic technologies have allowed traditional laboratory assays to be performed with high throughput and minimal reagents consumption [3], including antibiotics testing based on resazurin [4]. High-throughput has however been focused on large number of samples while replicating laboratory protocols, thus still requiring long incubation time.

The microfluidic chip presented in **Chapter 4** with surface modification introduced in **Chapter 5** allows time course curves to be monitored up to 5 minutes, in continuous fashion and for 50 to 100 droplets in parallel. Taking advantage of this, the hypothesis that the resazurin assay can be performed in minutes under non-conventional conditions was investigated. First, continuous mixing in moving droplets is anticipated to increase the resazurin reduction rate compared to static incubation in microplate or microfluidic. Second, temporal resolution on the order of 0.1s through the sample generation and incubation process enables faster kinetics than can be assayed with manual handling. Finally, fitting of initial reduction rates makes the measurement independent of starting fluorescence level and less prone to error compared to single fluorescence timepoint as it incorporates hundreds of measurement points. The effect of flow rate, initial resazurin concentration, cell density as well as growth phase on the resazurin reduction and apparent toxicity from heavy metals was assessed.

Pressure driven flow is first introduced to avoid flow oscillations from syringe pumps. An integrated magnetic stirrer is also added to generate logarithmic concentration gradients. Using this serial dilution, the following assay components are assessed for concentration dependence: resazurin, *E. faecalis*, and cationic metal toxicants. Heavy metals were used

as model toxicants to avoid partitioning into the oil phase at this stage. Five metals frequently found in wastewater were selected to cover a broad spectrum from potent and non-essential (Hg^{2+}) to essential with varying level of toxicity (Cu^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+}). Versatility of the microfluidic platform is demonstrated with yeast as an alternative microorganism potentially useful as low-cost bioreporter due to its availability as Baker's yeast, long storage capabilities and minimal culture requirements.

6.2 Experimental Methods

6.2.1 Materials and Chemicals

Hexadecane, 99% and n-Dodecyltrichlorosilane (DTS), 96% were purchased from Alfa Aesar, USA. Colloidal silica Ludox TM-50, resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), resorufin (7-Hydroxy-3H-phenoxazin-3-one sodium salt), CuSO_4 , ZnCl_2 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, HgCl_2 , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and brain heart infusion (BHI) broth were purchased from Sigma Aldrich, Singapore. Yeast extract, peptone and (D+) glucose were purchased from VWR, Singapore. Deionized water was used in all experiments. PMMA sheets 3 mm thick were obtained from Marga Cipta, Indonesia.

6.2.2 Fabrication of PMMA Microfluidic Chip with Integrated Stirrer

Microfluidic chips were made by milling 3 mm thick PMMA sheets followed by solvent vapor bonding as described by Ogilvie et al. [5]. The design shown in **Figure 6.1** was generated in Autodesk Fusion 360 and milled 100 μm deep on a desktop CNC mill (STEP-CRAFT-2/300, Stepcraft). The main channel and inlets were 700 μm and 300 μm wide respectively. Cylindrical chambers, $\varnothing 1.1$ mm, 1-1.4 mm long or $\varnothing 3$ mm x 6.5 mm long were also milled and a magnetic stirrer was inserted before bonding. A standard PTFE coated stirrer $\varnothing 2$ mm x 5 mm long was used for the larger chamber and could only wiggle, leaving ~ 30 μL for the liquid. For the smaller chamber, micro-magnets $\varnothing 0.8$ mm x 0.6 mm long were fabricated according to the scheme depicted in **Figure 6.2**. Neodymium magnets were crushed into powder, mixed at 70% weight ratio with epoxy (EasyCast Clear Casting Epoxy) and injected in FEP tubing with 800 μm internal diameter. After curing magnetic

bar or disc were obtained by cutting the tubing to the desired length. Finally, channels were coated with SiNPs and silanized with DTS as described in **section 5.2** except the stirred chamber was filled with paraffin wax prior to silanization to keep it hydrophilic. The magnetic stirrer was actuated by placing the microfluidic assembly on a stirrer plate at 800 rpm (UC151, Stuart).

Figure 6.1 Microfluidic design for serial dilution with an integrated magnetic stirrer in 1.1 mm diameter chamber. Reservoirs are pressurized at the same pressure and ratios between flow rates is pre-determined by the resistance of the tubings. Logarithmic concentration gradients can be generated with simple shutoff valves.

Figure 6.2 Fabrication sub-millimetre magnetic stirrer bar or disk from magnetic powder cast in epoxy.

6.2.3 Pressure Driven Flow and Droplet Velocity Controlled by Video Feedback

The same setup with camera and LEDs as described in **section 4.2** was used but syringe pumps (except sample loading) were replaced by pressurized 15 mL centrifuge tubes as illustrated in **Figure 6.1**. On-chip serial dilution was achieved by running a diluent stream through a stirred chamber that was pre-loaded with a sample using a syringe pump (Mitos Duo XS-Pump, Dolomite). The outgoing stream with exponentially decaying concentration was immediately segmented into droplets by a continuous oil phase and up to two additional reagents (aqueous inlets 3 and 4) were injected in continuous fashion. Liquids were delivered from pressurized reservoirs and flow was turned on or off by manual rotary valves (P-732, Upchurch scientific) except the diluent stream that was automated by computer controlled valve from the second channel of the pump (valve only, the syringe was removed). Flow rates ratios were set by fixed length of FEP tubings acting as hydraulic resistors. Tubing lengths and diameters were determined from electric circuit analogy [6]:

$$\Delta P = R_h Q \quad (\text{eq. 6.1})$$

Where ΔP is the pressure differential, Q the flow rate and R_h the hydraulic resistance given by the viscosity of the liquid μ and, for channel of length L and circular cross-section with radius R :

$$R_h = \frac{8\mu L}{R^4} \quad (\text{eq. 6.2})$$

And for rectangular cross section of height h and width w

$$R_h \approx \frac{12\mu L}{wh^3(1 - 0.630 h/w)} \quad (\text{eq. 6.3})$$

Figure 6.3 (A) Channel layout in **Figure 6.1** represented by its electric analogy. The resistance of the inlets and connecting channels can be neglected if the resistance of the tubings are much larger, thus making the ratio between inlets independent of ΔP and Q (i.e. ΔP is the same across all R_t). (B) Variations of the incubation channel resistance (R_{inc}) is compensated by adjusting the driving pressure with video feedback to maintain a constant incubation time.

Using the electric analogy, the channel layout in **Figure 6.1** is equivalent to the circuit in **Figure 6.3A**. Since the sample was loaded separately, it was not considered in the calculations. The circuit can be further simplified if the resistance of the tubings is much greater than the interconnecting channels and inlets. With this approximation, the pressure differential is the same for all reagents and flow rate ratios is inversely proportional to the resistance of the tubings. Tubing length and diameter were chosen to generate 2:1 oil to droplet ratio with 20% of the final droplet volume added at both injection inlets as summarized in **Table 6-1**.

Table 6-1 Targeted flow rate ratios between the different reagents and tubing length and diameter selected to achieve it according to **Equation 6.2**.

	Hexadecane	Diluent (aq 2)	Aqueous 3	Aqueous 4
Targeted flow rate ratios	2	1 (aqueous combined)		
		0.6	0.2	0.2
Tubing length (cm)	200	50	150	150
Tubing diameter (μm)	250	100	100	100
Hydrodynamic resistance* (Pa s m^{-3})	3.91E12	7.64E12 (aqueous combined)		
		1.27E13	3.82E13	3.82E13
Dead volume (μL)	98.2	3.9	11.8	11.8

* viscosities of pure hexadecane (0.003 Pa s [7]) and water (0.001 Pa s [8]) used for estimation.

While the ratios between flow rates were fixed, the absolute flow rate was varied by adjusting the driving pressure generated by a peristaltic pump (KCS-N14-SB6A, KSP1302, Kamoer) and pressure sensor (24PCCFA6D, Honeywell), both controlled by a digital I/O card (Analog discovery, Digilent). The pressure was adjusted to maintain a fixed incubation time using the estimated average droplet velocity from the video as feedback as illustrated in **Figure 6.3B**, because hydraulic resistance of the incubation channel varies in real-time.

6.2.4 Injection Volumes and Concentration Gradient Validation

The concentration gradient was calibrated via fluorescence measurements of resorufin dilutions. Resorufin (10 μM in PBS) was loaded as sample (aq. 1), PBS was used as diluent (aq. 2) and for additional reagents injection as aqueous 3 and 4 (**Figure 6.1**). Injected volumes were estimated from the reduction in fluorescence intensity after each injection. Calibration curve for the serial dilution was obtained from the average intensity of the droplets in incubation channel. Dilution sequence was repeated at least 3 times to determine standard deviation. To measure low fluorescence intensities, the camera exposure time and analog gain respectively were increased from 15 ms and 0 under normal operating conditions to 100 and 3. A 580 nm long pass filter (LP580-30.5, MidOpt) was added to

reduce the scattered excitation light (530 nm) that saturated the detector, including the red layer under these acquisition settings.

6.2.5 Resazurin Reduction Kinetics by Bacteria and Yeast and Toxicant Inhibition Curves

A series of experiments was carried out where logarithmic gradients of either resazurin, microorganisms or toxicant were generated while the other two were injected at fixed concentration (aqueous inlet 3 and 4). *E. faecalis* OG1RF (ATCC® 47077™) grown to stationary phase in BHI broth was used in most experiments. Unless otherwise indicated, 10 μ M resazurin was used and bacteria were diluted to generate droplet with OD₆₀₀=0.1 and the reservoir was kept on ice to prevent growth while the chip was maintained at 37 °C. OD₆₀₀=0.1 was estimated at 3.7×10^7 cell mL⁻¹ by the acridine orange direct count method [9]. Toxicant inhibition curves were also measured for *Saccharomyces cerevisiae* (Baker yeast) regrown on yeast extract, peptone, dextrose (YPD) medium. Optical density was measured with 1 mm optical path (nanodrop 1000, Thermo Fisher) and normalized to 1cm.

6.2.6 Determination of Initial Resazurin Reduction Rates and Inhibition Curves Fitting

Fluorescence time course measurements of each droplets were acquired with a camera as detailed in **Chapter 4**. Initial resazurin conversion rates were obtained from linear fit of the fluorescence signals from 5 s after mixing organisms and resazurin until 10% of the resazurin was converted or the maximum incubation time was reached as illustrated in **Figure 6.4A**. Inhibition curves were obtained by fitting the conversion rates as function of toxicant concentration with 4 parameters logistic function (4PL) as shown in **Figure 6.4B**. When the lower plateau (complete inhibition) was not covered by the concentration gradient, the average rate in negative control (without microorganisms) was used as constraint. Reported standard deviations correspond to at least 3 independent dilution series.

Figure 6.4 (A) Example of determination of initial rates for the resazurin reduction. Rate are determined from the slope of linear fit of the time course curves until 10% of the resazurin is converted or the maximum incubation time is reached. (B) Corresponding rates are fitted with a 4PL model to determine half inhibitory concentrations.

6.3 Results and Discussion

6.3.1 Reagents Injection Volumes is Set by the Hydraulic Resistance of the Inlets

Pressure driven flow is pulseless and more responsive compared to syringe pumps [10] and therefore more adapted to rapid transition between reagents needed in our application. Most versatile pressure driven pumps include individual pressure regulators and flow sensors for each channel which makes them as complex and as expensive as syringe pumps. However, by designing it specifically for serial dilution of a sample with injection of reagents at fixed volumetric ratios, simplifications can be made. Firstly, a single pressure source was used and ratios between reagents was permanently set by the resistance of the tubings. This approach eliminates the possibility of backflow and is inherently more robust than varying the driving pressure of individual reservoirs. Secondly, droplets velocity is readily available from the camera acquisition and can be used as feedback to maintain a constant incubation time without additional flow sensors.

Figure 6.5 Validation of injected volumes from fluorescence of resorufin droplets injected with PBS at aqueous inlets 3 and 4. Hydraulic resistors were designed to injected 20% of the final droplet volume at inlets 3 and 4.

The injected volumes were estimated by the reduction of fluorescence intensity after adding buffer to resorufin droplets as shown in **Figure 6.5**. Injections were reproducible with coefficient of variation of 1.2%. Volumes injected at the last inlet (aq. 4) were systematically 2% higher than predicted because of the resistance of the channel between the inlets was neglected in calculations (**Figure 6.3**). This small systematic error was accounted for when preparing the stock solutions but could also be reduced by increasing the tubing length at the cost of higher driving pressure. The driving pressure was 300 to 400 mBar under normal conditions and is typically limited to 1 bar by the reservoirs. Higher pressure is not practical anyway as gas solubility would be substantially higher in reservoir than on chip, causing bubbles to appear. The proposed design is a compromised between lowering the driving pressure and minimizing dependency of the injected volumes to the total flow rates ($R_t \gg R_{link}$). Finally, flow rate dependency to the sample viscosity does

not affect the injected volumes as it is only loaded in the stirred chamber before each dilution sequence.

6.3.2 Serial Dilution Profile is Determined by the Stirred Chamber to Droplet Volumetric Ratio

Concentrations gradients independent of diffusion coefficient are needed for unknown sample but are difficult to obtain without active mixing. Droplet trap in microfluidic channel have been used for serial dilution (**Figure 2.2E**), however the droplet content is not fully mixed between 2 merge and split operations [11] or requires large amount of oil between droplets to passively stir the trap [12]. Gielen et al. created gradients by generating droplets from an open micro well continuously mixed with a stirrer bar (**Figure 2.2F**) [13]. Here we integrate a magnetic stirrer directly on chip as active mixing element. Aqueous stream coming out from the stirred chamber is immediately split in droplet thus removing time delays and minimizing diffuse effect. Assuming complete mixing, the concentration in the chamber as function of time $C(t)$ is given by:

$$C(t) = C_0 e^{-\frac{Q}{V_c} t} \quad (\text{eq. 6.4})$$

Where C_0 is the concentration at $t=0$, Q the volumetric flow rate and V_c the volume of the mixing chamber. Given the number of droplets generated $x = \frac{Q}{V_d} t$ where V_d is the droplet volume, the concentration becomes:

$$C(x) = C_0 e^{-\frac{x}{\frac{V_c}{V_d}}} \quad (\text{eq. 6.5})$$

When $V_c \gg V_d$ (i.e. when concentration in the chamber does not change significantly while a droplet is formed) this is also the concentration of the droplet. Microfluidic chips were made with 1 and 30 μL chambers and the gradient was characterized by dilution of fluorescent resorufin using PBS as diluent.

Figure 6.6 Calibration of on-chip concentration gradient with serial dilution of resorufin in (A) $\sim 1 \mu L$ and (B) $\sim 30 \mu L$ stirred chambers. Error bars represent standard deviation of 4 dilution sequences.

As shown in **Figure 6.6**, when the stirrer was turned on, droplets fluorescence intensity followed an exponential decay as predicted by **Equation 6.5**. Different concentration profile was also obtained when the stirrer was switched off. At first, the sample was pushed out by advection and first few droplets had the maximum concentration. At longer timescale, solute remaining in the chamber was eventually cleared by diffusion [14]. While repeatable this regime is undesirable as the profile depends on diffusion coefficients which is unknown for real sample. When plotted in log scale as in **Figure 6.7**, a plateau corresponding to the background level of the camera is observed and droplet concentration cannot be quantified. Increasing the overall gain by a factor 20 raised the background level despite an additional long pass filter and only 5 times lower concentrations could be measured (background level is at approx. 10 nM resorufin). Measurements are limited by the limit of detection but it confirms that the gradient covers at least 3 orders of magnitude with less than 5% variation. Concentration is likely to continue to decrease following an exponential decay albeit with increasing coefficient of variation as commonly observed in serial dilution.

Resolution of the gradient can be adjusted in predictable manner by adjusting the chamber to droplet volumetric ratio. High resolution provides better accuracy for determination of EC50 from dose response curves, especially for sharp transitions where the region of

interest is only covered by 1 or 2 points with traditional methods. However, higher resolution (i.e. more points) takes more time. In this setup, droplets generation rate is restricted by the incubation time. Time course curves are typically recorded up to 3 minutes, corresponding to a droplet generation frequency of 0.5 Hz. Under these conditions, a single dilution sequence in **Figure 6.6 A and B** takes 3 min and 75 min respectively. For continuous toxicity monitoring, this time corresponds to the sampling interval, thus a 1 μL chamber was used in the remaining experiments as a good compromise between time and resolution.

Figure 6.7 Calibration of on-chip serial dilution in log scale. An apparent plateau is reached when fluorescence signal is near the background level of the camera. Concentration continue to decrease exponentially, covering at least 3 order of magnitude as confirmed by the lower limit of detection at higher gain and longer acquisition time.

6.3.3 Continuous Mixing in Moving Droplets Increases Resazurin Reduction Rate

It was hypothesized in previous chapter that the resazurin reduction was faster and more sensitive to toxicants in droplet format than on microplate because of enhanced mixing. We further investigated the effect of mixing by measuring the resazurin reduction rate while varying the droplet velocity (i.e. incubation time). Resazurin was injected in droplets

containing *E. faecalis* without any toxicant to a final concentration of 10 μM and $\text{OD}_{600} = 0.1$ respectively. As shown in **Figure 6.8**, the resazurin reduction rate first increased rapidly with increasing droplet velocity before saturating at about 3 $\mu\text{M min}^{-1}$. Unlike laminar flow in single phase, complex recirculating streamline are formed in moving droplet thus enhancing mixing [15]. Complete mixing typically requires the droplets to move 10-30 times their length as shown experimentally [16] and by simulations [17] and therefore directly related to their velocity. Resazurin reduction is heterogenous as it only occurs inside the cells [18] and continuous mixing can mitigate or prevent local depletion of the substrate. Saturation of the reduction rate is consistent with transition from a diffusion limited regime to kinetic limited regime above 4 mm s^{-1} . Consequently, the assay performed above this critical velocity not only reduces the detection time but also increases the sensitivity towards toxic inhibition as diffusion is no longer a limiting factor. A velocity of 4 mm s^{-1} corresponding to incubation for 3 minutes was used in remaining experiments.

Figure 6.8 Resazurin reduction to resorufin by *E. faecalis* in droplets moving at different velocities. (A) Time course curves averaged over 30 droplets. Reagents injection is less repeatable at lower velocities resulting in the larger standard deviations observed. (B) Fitted initial rates as function of droplet velocity.

6.3.4 Resorufin to Dihydroresorufin Conversion is Not Observed

Two-hour long incubation time on microplate revealed that the secondary reduction to dihydroresorufin could potentially be a more sensitive indicator albeit with slower kinetics (**Figure 5.13**). The secondary reaction was not observed in microfluidic due to the shorter timescale. Resorufin was injected directly instead of resazurin as shown in **Figure 6.9**. Even starting directly from resorufin, no reduction was observed within 3 minutes. Unlike reaction from resazurin to resorufin which is irreversible, resorufin to dihydroresorufin can be reversed by the presence of oxygen [19]. A delayed reduction in fluorescence intensity could be due to the time taken by facultative bacteria to deplete the dissolved oxygen. In summary, the reduction of resorufin to dihydroresorufin is an unsuitable viability indicator for real-time toxicant detection. It also signifies that under our test conditions, dihydroresorufin conversion can be neglected in the following analysis of the resazurin kinetics.

Figure 6.9 Resorufin injected in *E. faecalis* droplets instead of resazurin. No reduction to non-fluorescent di-hydroresorufin is observed over 3 min.

6.3.5 Apparent Toxicity Increases with Decreasing Bacterial Density

Droplets containing serial dilution of *E. faecalis* harvested in stationary phase, 0 or 6 mg L⁻¹ Cu²⁺ and 10 μM resazurin were generated to assess the effect of bacterial density on

apparent toxicity. As shown in **Figure 6.10A**, the resazurin conversion rate varied linearly with bacterial density in range ~ 0.01 - 0.25 , estimated as bacterial density from 3.7×10^6 to 9.2×10^8 cell mL^{-1} . When copper was added, the apparent toxicity increased at lower bacterial density down to 0.01 OD_{600} as illustrated in **Figure 6.10B**. Increase of apparent toxicity is expected at lower bacterial density because of the higher toxicant to biomass ratio. This is however limited by the poor signal to noise ratio when approaching the background signal and the short 3 minutes incubation on microfluidic. The incubation time cannot be increased by changing the length of the incubation channel because of the limited space available. Meanwhile, increasing the incubation time by reducing the flow rate will not result in better signal to noise ratio because of slower kinetics due to less efficient mixing as discussed above. An optical density of 0.1 is therefore a good compromise between linearity of the response and minimizing the limit of detection.

Figure 6.10 (A) Reduction rate of $10 \mu\text{M}$ resazurin as function of bacterial density. (B) Effect of bacterial density on apparent copper toxicity.

6.3.6 More Than One Enzyme Can Be Responsible for *in-vivo* Reduction of Resazurin

Resazurin concentration can have a significant impact on the apparent toxicity but there is no consensus in literature. Concentrations ranging from $6 \mu\text{M}$ to 3mM have been reported [20-21] while Manufacturer instructions for Alamar blue generate samples with $44 \mu\text{M}$

resazurin [18]. Justifications, when given, are usually maximization of the fluorescence signal or linearity with respect to cell density. Inner filter effects from concentrated resazurin solution (e.g. above 34 μM for 1 cm optical path [22]) are also widely overlooked. Instead we propose to base the choice of resazurin concentration on its reduction kinetics. In order to detect potential competitive, non-competitive and uncompetitive inhibitors the substrate (i.e. resazurin) should be equal to the Michaelis constant K_m of the enzyme involved [23]. Droplets containing serial dilution of resazurin and fixed density of *E. faecalis* were generated and initial reduction rates were calculated from fluorescence time course curves to measure the apparent Michaelis constant. As shown in **Figure 6.11A** the velocity (i.e. resazurin reduction rate) increased sharply at low substrate concentration and then almost linearly at higher substrate concentrations, thus deviating from Michaelis-Menten (MM) model. Departure from linearity is evident in Lineweaver–Burk plot as displayed in **Figure 6.11B** and can indicate the presence of multiple enzymes with different substrate affinities [24]. Total velocity of two enzymes converting the same substrate in parallel is given by the summation of two MM processes:

$$v = \frac{[S]V_{max1}}{K_{m1} + [S]} + \frac{[S]V_{max2}}{K_{m2} + [S]} \quad (\text{eq. 6.6})$$

K_{m1} and K_{m2} should differ by at least a factor 20 for deviation to be apparent [25]. Experimental velocity profiles are compatible with this 2 enzymes model, suggesting the presence of two or more enzymes able to reduce resazurin, of which two have substantially different K_m . It should be noted that estimated $K_{m2}=140 \mu\text{M}$ was extrapolated and higher substrate concentrations could not be covered because of limited solubility of the resorufin product ($\sim 10 \mu\text{M}$ in PBS). At least two enzymes, dihydrolipoamine dehydrogenase and NADH dehydrogenase have separately been shown to reduce resazurin *in vitro*, but exact enzymes primarily responsible for resazurin reduction *in-vivo* are still up for debate [18]. Another common deviation from MM model for *in-vivo* kinetics is due to diffusion limitations across the cell membrane [26] and around membrane-bound enzymes [27]. It is however unlikely as droplets are continuously mixed and it should deviate towards a more linear curve compared to MM model, including in the low substrate region. Regardless, the

selection of the optimal resazurin concentration for toxicity assay is not straightforward because of deviation from MM model and is likely to be organism dependent.

Figure 6.11 (A) Resazurin reduction rate as function of substrate concentration for different *E. faecalis* densities. Dashed lines represent fitting of single enzyme Michaelis-Menten model and solid lines global fitting with 2 enzyme model and shared K_m . (B) Lineweaver–Burk representation highlighting deviation from Michaelis-Menten model (i.e. non-linearity).

6.3.7 Copper and Mercury Inhibit the Resazurin reduction by Different Pathways

The same kinetics study was repeated with bacteria at OD_{600} of 0.1 and with addition of mercury and copper at partially inhibitory concentrations. As shown in **Figure 6.12** apparent inhibition by copper increased along with substrate concentration while inhibition by mercury was predominant at low substrate concentration. The copper and mercury cations display aspects of non-competitive and competitive inhibitions mechanisms, respectively. Reported mechanism for mercury inhibition primarily involve non-competitive inhibition by means of covalent attachment to thiols of cysteine amino acids [28]. However more complex mixed inhibition mechanisms as for instance simultaneous competitive and non-competitive inhibition to Proteinase K have been observed [29]. Competitive inhibition by mercury is unlikely but cannot be ruled out with the given dataset. Strong deviation from MM model warrants other alternatives to be considered. For instance, the two-enzyme model presented above can adequately describe the trend observed if

mercury and copper preferentially inhibit different enzymes with low and high K_m constants respectively. Regardless of mechanism, these findings highlight how resazurin concentration gradients can lead to unique toxicant profiles.

Figure 6.12 Inhibition of resazurin kinetics by copper and mercury. *E. faecalis* at OD₆₀₀ 0.1 was used.

6.3.8 Apparent Copper Toxicity Increases in The Stationary Phase of *E. faecalis*

Standard assay protocols recommend using cells in the exponential stage of growth [2]. This however poses a problem for continuous toxicity monitoring because long term maintenance of cell in log phase requires a chemostat, thus introducing additional reagents, wastes and overall complexity. The impact of growth phase on apparent toxicity was assessed by continuously generating droplet dilution sequence of copper every ~7.5 min with sequential injection of growing *E. faecalis* and resazurin to a final concentration of 10 μM. The main difference with previous experiments was that bacteria injection was started immediately after inoculation and the reservoir was heated at 37 °C after 2.5 hours at room temperature. Examples of inhibition curves obtained at different time are shown in **Figure**

6.12 and **Figure 6.13** corresponding EC50 and maximum asymptotes over time are shown in **Figure 6.14**.

Figure 6.13 Examples of copper inhibition curves obtained at different time during the growth of *E. faecalis*.

The resazurin reduction rate (i.e. maximum asymptote) which is affected by the number of bacteria increased after an initial lag phase. The reduction rate can however also be affected by the metabolism of individual cells. It was particularly obvious at the 5.8 hour mark where it dropped within minutes. This change was so rapid that the rate substantially changed during a single copper dilution cycle as shown in **Figure 6.13** at 5.8 hours. Concurrently copper became apparently less toxic as the log phase progressed and the EC50 increased by nearly 2 orders of magnitude to 166 mg/L compared to 2 mg/L in lag phase. As addressed in previous section, it can only be partially attributed to an increase of biomass to toxicant ratio. Finally, the EC50 dropped to 10 mg/L in the late log-stationary phase, making copper apparently more toxic than in early log phase. Similar trend has been

reported by Teitzel et al. from viable plate count of planktonic *pseudomonas aeruginosa* harvested in log and stationary phases and exposed to copper [30]. They speculated that it could be due to enhanced heavy metal efflux in growing cells.

Figure 6.14 Real-time copper toxicity monitoring of growing *E. faecalis*. (Top) The apparent EC50 and (Bottom) maximum asymptote corresponding to resazurin conversion rates in control were estimated from 4PL fit of inhibition curves (**Figure 6.12**). Error bars represent fitting errors.

6.3.9 Ratio Between Enzymes Reducing Resazurin Rapidly Changes During Late Log Phase

To gain more insight on the growth phase dependence previously observed, the experiment was repeated with serial dilution of resazurin instead of copper to obtain substrate dependent kinetics as function of the growth phase. Optical density was also monitored to help distinguish between contributions to the overall reduction rate from the number of bacteria and from variation in metabolic activity. Overall the resazurin reduction rate increased as bacteria grew but the shape changed from quasi-linear to hyperbolic and back

towards linear as shown in **Figure 6.15**. Curves were globally fitted with **Equation 6.6** for 2 enzyme kinetics with shared K_m . Note that this assumes that enzyme concentration related to V_{max1} , V_{max2} changes but not their Michaelis constants. This assumption is necessary because fitting individual curves with 4 parameters results in overparameterization for more linear curves. Maximum velocities are plotted as function of culture time in **Figure 6.16A**. According to this model, concentration of both enzymes increased with bacterial growth but their ratio varied drastically. The enzyme with the lower Michaelis constant $K_{m1} \approx 3 \mu\text{M}$ increased during early log phase before suddenly dropping at the 4-hour mark. The other enzyme increased monotonically, following the bacterial density trend (**Figure 6.16B**), albeit not proportionally, because of non-linearity at high bacterial density (**Figure 6.10**).

Figure 6.15 Example of resazurin kinetics measured at different time during *E. faecalis* growth. Dashed lines represent global fitting with 2 enzyme model and shared K_m .

These results along with previous observation that copper preferentially inhibit the enzyme with higher K_m (**Figure 6.12**) suggest that the apparent increase in resistance to copper during the early log phase is due to variation in enzyme contribution rather than heavy metal efflux. At 10 μM resazurin, the transient increase in enzyme concentration not inhibited by copper

had a high contribution to the overall resazurin reduction rate thus masking the inhibition of the other enzyme. When its contribution suddenly dropped, inhibition of the other enzyme became apparent again. Increasing the resazurin concentrations is expected to make the assay more sensitive to copper during the log-phase but have an opposite effect on mercury. For general toxicity test, a balance between these two model toxicants is needed. Furthermore, for continuous toxicity monitoring, bacteria in stationary phase are preferable because they are easier to maintain and introduce less variability with respect to harvesting time. For these reasons, 10 μM resazurin and bacteria in stationary phase were chosen for toxicity experiments.

Figure 6.16 *E. faecalis* growth monitoring. (A) Maximum velocities from fitting kinetics in **Figure 6.15** with a two-enzyme model. (B) optical density.

6.3.10 The Resazurin Assay with *E. faecalis* in Droplet Format is Oversensitive Towards Copper and Zinc Compared to Reported Cytotoxicity

Figure 6.17 Inhibition of *E. faecalis* resazurin reduction by heavy metals. (A) Mercury. (B) Copper. (C) Zinc. (D) Nickel. (E) Cobalt, interference with resazurin or resorufin were observed above 54 mg L⁻¹ as well as precipitates moving in the droplets.

Once bacterial growth phase, density and resazurin concentration were optimized, inhibition curves were measured separately for 5 heavy metals, zinc, cobalt, copper, mercury and nickel. Droplets containing serial dilution of the metals (500 mg/L stock) were generated followed by sequential injections of *E. faecalis* and resazurin. Some of the metals precipitated when resazurin in PBS was injected, likely because a change of solubility induce by the buffer [31]. In the case of cobalt, precipitate formation came alongside with interference with resazurin or resorufin impurities. Therefore, the corresponding portion of the data was not used for fitting. Too much precipitates were formed with Hg^{2+} , Cu^{2+} and Zn^{2+} resulting in droplet pinning and curve could not be recorded. Stock concentration were adjusted accordingly: Cu^{2+} 100 mg L⁻¹, Zn^{2+} 50 mg L⁻¹ and Hg^{2+} 25 mg L⁻¹. The apparent toxicity ranking based on EC50 was $\text{Hg}^{2+} > \text{Zn}^{2+} > \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+}$ as shown in **Figure 6.17**. In case of Hg^{2+} and Zn^{2+} a plateau was reached before complete inhibition in which case the apparent EC50 is reported. This could be explained by the formation of precipitates which are less toxic than their ion counterpart or as discussed above, inhibition of only some of the enzymes reducing resazurin.

The EC80 values obtained from the resazurin assay in microfluidic format are compared to MICs reported in literature and were close for Hg^{2+} as shown in **Table 6-2**. Co^{2+} and Ni^{2+} EC80 were substantially higher but are only extrapolation because complete inhibition was not reached in the concentration range tested. EC80s for Cu^{2+} and Zn^{2+} are one to two orders of magnitude lower than their reported MICs. This is in contradiction with good correlation between resazurin reduction and bacterial growth previously reported with long pre-incubation time (24 h) [32]. It is generally assumed that inhibition of resazurin reduction indicates cytotoxicity. It has however been shown that some compound such as dicumarol can specifically inhibit enzymes reducing resazurin without being cytotoxic [33]. In this study, inhibition of two different enzymes could be distinguished which suggests inhibition of specific enzymes reducing resazurin, or at least inhibition of distinct metabolic pathways rather than indicating an overall decrease in cell viability. It is possible that short exposure time in droplet format (30 s) accentuates the effect of metabolic activity on resazurin reduction without necessarily reflecting cytotoxicity. Although Cu^{2+} and Zn^{2+} EC20s are

far below reported MICs, important reorganization of metabolic pathways occurs at these concentrations which might be reflected by the resazurin reduction [34-36].

Table 6-2 Comparison of heavy metals toxicity to *E. faecalis* from resazurin reduction EC80 in microfluidic droplet format (3 min) and literature reported MIC based on growth inhibition on agar plates (14-24h). Concentration are in (mg L⁻¹)

	Hg ²⁺	Zn ²⁺	Cu ²⁺	Ni ²⁺	Co ²⁺
Resazurin EC80 (this work)	2.6	5.1	28.6	1428	9000
MIC	2 [37], 10 [38]	130 [39], 654 [38]	127, 1016 [39], 190 [38]	350 [38]	236 [38]

For the purpose of detecting toxicants in water, the microorganisms act as a canary in a coal mine. Therefore, their fate is not essential as long as the response is repeatable and the dynamic range of response matches the application needs. *E. faecalis* would be a poor indicator for drinking water safety but has a good potential as early warning for wastewater bioreactors. For instance Chen et al. previously reported that the addition of Ni²⁺ or Co²⁺ to anaerobic sludge at 5 mg L⁻¹ can stimulate the methane production while 250 mg L⁻¹ are inhibitory which fits well with the detection range from *E. faecalis* [40]. A drawback of this approach is that an extensive validation, including different type and combinations of toxicants will be needed to ensure overall correlation between both.

6.3.11 Resazurin Reduction in Yeast is inhibited by Copper but Increased by Zinc and Nickel

Due to its availability and ease of culture, commercially available Baker's yeast could be another useful bioreporter that requires minimal preparation. Hg²⁺, Cu²⁺, Co²⁺, Ni²⁺, Zn²⁺ were tested with *Saccharomyces cerevisiae*. Droplets containing serial dilution of the metals were generated followed by sequential injections of yeast and resazurin. Yeast optical density in droplets was 0.5 and resulted in 4 to 5 times slower resazurin reduction compared to *E. faecalis*. The apparent toxicity ranking based on EC50 was Cu²⁺ > Hg²⁺ > Co²⁺ as shown in **Figure 6.18**. Cu²⁺ was five times more inhibitory to yeast than *E. faecalis*

while the others were less. Zn^{2+} above 8 mg L^{-1} increased the resazurin reduction rate while with Ni^{2+} the reduction rate peaked at 100 mg L^{-1} . An increase in rate could be due to a variety of reasons, including activation of metal dependent enzymes [41-42], impaired mitochondrial oxidation [43] or increase in cellular activity as part of toxicity resistance mechanism [44-45]. Stimulation is plausible, at least for zinc at tens of mg L^{-1} [46-47], in which case the assay might allow determination of heavy metal stimulations. Complementary assays are needed to verify if the increased rate is sustained or temporary as the result of toxicity.

Saccharomyces cerevisiae is widely studied and therefore a variety of data are available for comparison. However as presented in **Table 6-3**, reported EC50 vary drastically among laboratories, even when similar methods are used. A possible reason for these discrepancies is a difference in bioavailable form of the metals depending on the medium used [31]. Noticeably, a direct comparison is possible with the microplate toxicity screen for fungicide based on *Saccharomyces cerevisiae* and resazurin developed by Fai et al. [48]. They measured virtually the same EC50 for Cu^{2+} albeit with a total incubation time of 70 minutes whereas it only takes 3 in droplet format. The faster detection in droplets can be attributed not only to enhanced mixing but also fitting of initial rates (**Figure 6.4**) allowing robust estimation, even at suboptimal fluorescence intensities.

Table 6-3 Comparison of heavy metals toxicity to *Saccharomyces cerevisiae* from resazurin reduction EC50 in microfluidic droplet format (3 min) and literature reported data. Concentration are in (mg L^{-1}). [48]

	Cu^{2+}	Hg^{2+}	Co^{2+}	Ni^{2+}	Zn^{2+}
Resazurin (this work)	2	33	>40	>300, rate variation	>30, rate increased
Tetrazolium [49]	79	101		6151	163
Tetrazolium [50]	5.6	0.8		20	19
Optical density [51]		15	23	117	164
Optical density [52]	11.4				75
Resazurin [48]	1.5				
Optical density [48]	604

Figure 6.18 Inhibition of *E. faecalis* resazurin reduction by heavy metals. (A) Mercury. (B) Copper. (C) Zinc. (D) Nickel. (E) Cobalt.

6.4 Conclusion

Herein we presented and discussed the resazurin assay performed under alternative conditions from traditional laboratory procedures to reduce the response time, including short toxicant exposure (< 30 s) and stationary phase bacteria at high density (3×10^7 cell mL^{-1}) for rapid toxicant detection in microfluidic format. The microfluidic platform was designed to record fluorescence time course kinetics at 10 sample s^{-1} , allowing robust fitting of initial rates instead of single timepoint. We established that continuous mixing in moving droplets increases the resazurin reduction rate and suggested that it made the assay more sensitive than on microplate by removing diffusion limitations. We also demonstrated that under < 5 min incubation time, the resazurin reduction cannot be simply related to the number of viable cells. Its reduction likely involves 2 or more different enzymes with Michaelis constants differing by a factor of more than 20. Consequently, it was demonstrated using copper and mercury ions as models, that the choice of resazurin concentration and growth phase can alter the toxicity profile in toxicant dependent manner. These results imply that in this format, it is not possible to predict cell viability from resazurin inhibition, at least not without a better understanding of the inhibition mechanisms. Unlike in antibiotic screening however, the fate of the bioreporter is not crucial for water testing and the apparent inhibition could be used as indicator of toxicity after extensive validation with a wider range and combinations of toxicants.

The microfluidic version of the resazurin assay is automated and works in continuous mode which makes it a potential alternative to genetically engineered bioreporters for water toxicity monitoring. It does not give the option to target specific compounds, but for general toxicity it presents several advantages. 1) The response time is less than 5 minutes compared to 0.5 to 2 hours for engineered bioreporters [53]. 2) Whole effluent toxicity is readily quantifiable by on-chip serial dilution [54]. 3) It can be adapted to a variety of reporting organisms without genetic modifications. 4) Like in traditional bioassays, fresh samples are independent of previous exposure to toxicants and matrix interferences is detected in control samples. Continuous reagents consumption can be a drawback but resazurin is cheap and the continuous oil phase is easily recycled. Overall complexity can

also be a limitation depending on application. The major limitation in its current form is however the partitioning of hydrophobic compounds in the continuous hexadecane phase which is addressed in the next chapter.

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Chapter 7

Fluorinated Continuous Phase

Previously partitioning of hydrophobic compounds in the oil phase resulted in underestimation of toxicity. To overcome and expand the toxicity assessment towards hydrophobic toxicants (LogP 1-4), surface modification combining chemistry and morphology is adapted to fluorocarbons. Stability towards biofouling or hydrolysis are discussed in terms of predicted repulsive Van der Waals interactions. Perfluorocarbons, hydrofluoroether and hexadecane are also quantitatively compared based on their PCP distribution coefficient versus PBS and their actual impact on measured EC50.

7.1 Introduction

Droplets are used as isolated microreactors in segmented-flow bioassays but the choice of continuous oil phase can severely undermine this idealized concept. This is particularly important for wastewater analysis where many relevant pollutants are hydrophobic and therefore likely to be extracted by the oil phase [1]. For instance, it was reported in **Chapter 5** that the apparent toxicity of PCP was at least ten times lower using droplet in hexadecane than in analogous microplate experiments. Extraction from and transfer between droplets can occur through phase partitioning and, when surfactants are involved, through reverse micelles or by direct transfer across lipid bi-layers. There has been no unequivocal evidence indicating that surfactant could act as a barrier limiting transfer across water-oil interface [2]. Therefore, in applications where droplets are not contacting each other, surfactants should be avoided to prevent transport enhancement which leaves us with partitioning as main transfer mechanism. Fluorinated oil phases have lower solubility for non-fluorinated molecules and minimize leakage from droplets compared to hydrocarbons or silicone oils [3-5]. A variety of fluorinated reagents have been used in droplet-based microfluidic and candidates that could replace hexadecane with similar viscosity are - illustrated in **Table 7-1**. Although all contain fluorinated chains, there could be considerable difference in terms of partitioning among them, the most obvious one being HFE-7500 which has a non-fluorinated group. Mazutis et al. reported that resorufin was exchanged between droplets 5 times faster in HFE-7500 than in FC-40 but droplets were in direct contact with 3% fluoro-surfactant thus left in question the contribution of partitioning [6].

Herein, we address the challenges faced to ensure wetting by fluorinated continuous instead of hydrocarbons, with the focus on strategies that avoid surfactants. Performances of the coating with respect to biofouling are also discussed for long term toxicity monitoring. Different continuous phases are also quantitatively compared based on distribution coefficients of PCP as well as their practical impact on EC50 estimation.

Table 7-1 Fluorinated liquids considered for segmented-flow microfluidic compared to hexadecane and comparison of PCP solubility with Hansen parameters and distance to PCP calculated according to **Equation 3.4**. Physical parameters at 25 °C.

Short name	FC-40	FC-43	FC-3283	FC-770	HFE-7500	Hexadecane
Structure
Name	mixture, perfluoro-di-n-butylmethylamine, Perfluorotributylamine	Perfluorotributylamine	Perfluorotripropylamine	octafluoro-4-(heptafluoropropyl)morpholine	3-Ethoxyperfluoro(2-methyl)hexane)	n-hexadecane
Refractive index	1.290	1.291	1.281	1.270	1.287	1.434
Dielectric constant	1.9	1.9	1.9	1.9	5.8	2.08
Viscosity (cP)	3.4	4.7	1.4	1.4	1.24	3
Density (g cm ⁻³)	1.85	1.86	1.82	1.793	1.614	0.77
Average Mw (g mol ⁻¹)	650	670	521	399	414	226
Hansen solubility parameter (MPa ^{0.5}) [δ _d , δ _p , δ _h]	-	[12.8, 0, 0.3] ref. [7]	[12.2, 1, 0.7] ref. [7]	-	[13.3, 2, 1] ref. [8]	[16.3, 0, 0] ref. [9]
Hansen distance to PCP, ref. [9] [21.5,6.9,12.8]	-	22.5	23.0	-	20.8	19.7

7.2 Experimental Methods

7.2.1 Materials and Chemicals

Hexadecane, 99%, n-Dodecyltrichlorosilane (DTS), 96%, and 1H,1H,2H,2H-perfluorodecyltrichlorosilane (FDTS), 96% were purchased from Alfa Aesar, USA Fluorocarbons (FC) FC-40, FC-43, FC-3283, FC-770 and hydrofluoroether (HFE) HFE-7500 were purchased from 3M, Singapore. Colloidal silica Ludox TM-50, resorufin (7-Hydroxy-3H-phenoxazin-3-one sodium salt), resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) and brain heart infusion (BHI) broth and pentachlorophenol (PCP) were purchased from Sigma Aldrich, Singapore. Deionized water was used in all experiments. PMMA sheets 3 mm thick were obtained from Marga Cipta, Indonesia.

7.2.2 Microfluidic Chip Fabrication

Microfluidic chips were made milling PMMA sheets followed by solvent vapor bonding as described by Ogilvie et al. [10]. The design and machining files were generated in Autodesk Fusion 360 and milled on a desktop CNC mill (STEP-CRAFT-2/300, Stepcraft, Germany). UV etching could not be used here because it results in different material properties of the surface and the bulk leading to different response to the solvent treatment described below.

7.2.3 PMMA Surface Modification

Some channels were coated with SiNPs and silanized with DTS as described in section 5.2. Silanization with FDTS instead of DTS was attempted with vapor and liquid phase deposition methods. For the vapor phase deposition, a microfluidic chip coated with SiNPs and an O₂ plasma treated glass slide as control were placed in a desiccator with 50 μ L of FDTS or hexane solution of FDTS [11]. Vacuum lines were connected directly to the desiccator and through one port of the chip so that desiccator could be evacuated rapidly without flow restriction from the microchannel. The desiccator was evacuated and left under vacuum for at least 2 hours. For the liquid phase methods, 0.5 and 0.1 % (v/v) FDTS

solutions were prepared in Schlenk flasks under nitrogen stream from hexane or FC-3283 that were pre-dried with 5 Å molecular sieves. After deposition, the channel was washed with the same solvent.

Channels with increased roughness were obtained by swelling the PMMA surface with 6:4 acetone:ethanol (v/v) followed by rapid precipitation in DI water before silica deposition. For characterization, PMMA slides, 1 cm x 1 cm were exposed to chloroform vapor for 4 minutes and kept in oven at 80 °C for 60 min to mimic the chip bonding procedure and treated in the same manner for 30, 60 and 120 s. Surface roughness was characterized by atomic force microscopy (AFM) (Cypher S, Asylum Research, USA) in attractive mode (i.e. non-contact) with a 160 kHz tip (NCSTR, Nanoworld, Switzerland). The scan area was 10 µm x 10 µm, and scan rate 0.5 Hz; the values reported are the average of 4 scans at different locations.

7.2.4 Microfluidic Device Operation

The same setup with camera and LEDs as described in section 4.2 was used with image processing parameters adjusted for fluorinated oils. Pressure driven flow and the concentration gradient method were utilized as described in section 6.2

7.2.5 PCP Distribution Coefficients

PCP was first dissolved at 10 g L⁻¹ in methanol and then diluted at least a 500X in Phosphate buffered saline (PBS) or hexadecane. For distribution coefficient, 750 µL of 20 mg L⁻¹ PCP in PBS was mixed with 750 µL of pure oil in triplicates, sonicated for 5 minutes followed by centrifugation for 5 minutes. 350 µL of each phase was immediately extracted for absorbance measurement (Agilent Cary, 50 UV-Vis). Calibration curves were obtained for PCP in PBS and hexadecane. Molar absorptivity was estimated from linear fittings using values in the linear range of the spectrometer. The 5 fluorinated oils used had the same peaks ratio as in hexadecane and were shifted by at most 3 nm, hence molar absorptivity in hexadecane was used to estimate PCP concentration in fluorinated oils.

Hansen solubility distance between PCP and liquids used as continuous phase were also calculated from available literature data per **Equation 3.4**.

7.2.6 Estimation of Hamaker Constants

Non-retarded Hamaker constants were estimated according to an approximation of the Lifshitz theory for two phases 1 and 2 interacting across a third medium 3 [12]:

$$A_{132} \approx \frac{3}{4}kT \left(\frac{\varepsilon_1 - \varepsilon_3}{\varepsilon_1 + \varepsilon_3} \right) \left(\frac{\varepsilon_2 - \varepsilon_3}{\varepsilon_2 + \varepsilon_3} \right) + \frac{3\hbar v_e}{8\sqrt{2}} \frac{(n_1^2 - n_3^2)(n_2^2 - n_3^2)}{(n_1^2 + n_3^2)^{1/2}(n_2^2 + n_3^2)^{1/2}\{(n_1^2 + n_3^2)^{1/2} + (n_2^2 + n_3^2)^{1/2}\}} \quad (\text{eq. 7.1})$$

Where ε_i , n_i and v_e are the dielectric permittivity at $\nu = 0$, the refractive index in the visible and the main electronic absorption frequency in the UV region respectively.

7.3 Results and Discussion

7.3.1 Vapor Versus Liquid Phase Deposition of Fluorinated Chlorosilane in Microchannel

Silanization of PMMA chips with fluorinated silanes was first attempted with the procedure based on SiNPs that was developed in **Chapter 5**, except DTS was replaced by FDTS. At 0.5% (v/v), FDTS is soluble in hexane but it resulted in white films and aggregates were systematically observed after an hour. Lowering the concentration to 0.1% or replacing hexane by FC-3283 prevented the formation of macroscopic white films but droplet generation and motion was irreproducible. As visible from the meniscus of the droplets in **Figure 7.1**, surface wetting was inhomogeneous and droplets appeared to drag against the walls. This “drag” caused the droplets to deform allowing the continuous phase to flow around them and in the worst case remained completely pinned. AFM analysis of sampled prepared in the same manner revealed the presence of ~100 nm particles on top

of the silica coating as shown in **Figure 7.2C**. In contrast, no difference between bare and silica silanized with DTS was visible at this scale.

Figure 7.1 Erratic flow of aqueous droplets in fluorinated oil in channels (700 μm wide) treated with FDTS. (A) Droplets shape is asymmetrical and move in stepwise fashion, indicating inhomogeneous surface wetting. (B) Partial pinning in some area slow down the droplets, allowing the continuous phase to flow around them, thus reducing the spacing. (C) Injection of water into resorufin droplet. Pinning at the injection junction causes droplet to break further down the channel, causing cross-contamination with subsequent sample and irreproducible injection volumes.

Moisture induced self-polymerization of trichlorosilanes is a well-known problem but a tricky one because some degree of moisture is required to form a complete monolayer [13]. Nanoscale aggregates result in heterogenous surface which increases contact angle hysteresis [14-16] and are consistent with the velocity mismatch between the droplets and continuous phase. Monolayers of FDTS have been shown to form in 5 min versus 90 min for DTS at the same concentration [17] which can explain why no aggregates were observed with DTS. Time to form aggregates also depends on the solvent used and the presence moisture [18]. The reaction time was reduced to try avoiding aggregates. To observe the change in surface wetting in-situ, hexadecane droplets were generated in FC-3283. Before adding FDTS, fluorocarbon droplets in hexadecane were generated on SiNP coated channels as shown in **Figure 7.3**. After 30 minutes, half the time used in in normal

procedure, wetting regime was reversed over the entire channel and the reaction was stopped. This however did not prevent pinning of water droplet in fluorocarbon suggesting aggregates were not avoided. Formation of aggregates are systematically reported in studies looking at surface morphology of fluorinated trichlorosilanes monolayer assembled from liquid phase [19]. Fluoroalkyl silanization of plasma activated PDMS microfluidic is common for generation of aqueous droplet in fluorinated oils [20]. Given the procedures are far less elaborated than for monolayer studies, formation of aggregates has probably been overlooked because addition of surfactants in previously reported literature. Subramanian et al. reported droplet generation without surfactant under dynamic conditions, in static conditions droplets come in contact with the walls ($Ca = 0$), and was remedied by surfactant addition which seems to corroborate our observations [21].

Figure 7.2 Representative AFM images of PMMA slides coated with one cycle of SiNPs, (A) without hydrophobic modification, (B) modified with DTS from solution, (C) modified with FDTS from solution (>100 nm objects were found on all scanned areas), (D) modified with FDTS by vacuum deposition.

Figure 7.3 Visualizing FDTD surface modification in real time. FC-770 droplets in hexadecane are generated on silica coated PMMA. Thirty minutes after adding FDTD to FC-770 phase, wetting is completely reversed and hexadecane droplets in FC-770 are formed.

Vapor phase deposition of FDTD was also attempted and no aggrate were observed as shown in **Figure 7.2D**. In contrast to liquid phase, polymerized silanes do not evaporate and therefore not deposit on the substrate. As a control, contact angle on a plasma treated glass slide placed in the same desiccator was $115 \pm 2^\circ$, which is consistent with value reported in literature for FDTD on flat surface [22]. Unfortunately, vapor deposition did not yield the intended result of hydrophobic surface. The channels remained hydrophilic and were preferentially wetted by the water phase, thus generating fluorocarbon droplets in water. Vapor deposition is limited by diffusion path and while it has been reported for aspect ratio up to 37 [23], our channel is over 90 cm long, 100 μm deep, corresponding to an aspect ratio of 9000. Directly connecting the outlet port of the chip to a vacuum line resulted in hydrophobic modification over the 2-3 centimetres closest to the FDTD source, but the rest of the channel remained hydrophilic.

7.3.2 Surface with Increased Roughness and DTS Coating Prevents Cassie-Wenzel Wetting Transition of Aqueous Droplets in Fluorocarbon Oil

Hydrocarbon coatings on smooth surface would result in partial wetting between fluorinated oil and water, however because our silica nanoparticles coating provides a roughened surface with a DTS monolayer free of aggregates, it was investigated for use with fluorocarbons. As shown in **Figure 7.4**, droplets could be generated at high capillary number ($Ca \approx 4.3\text{E-}4$, droplet velocity $\approx 33 \text{ cm min}^{-1}$). When the flow was stopped ($Ca=0$)

and resumed, droplets remained temporarily pinned but were eventually dislodged. New incoming droplets also slowed down and deformed at the locations of previously pinned droplets. This memory effect slowly vanished after several droplets passed over the area. Droplet pinning can be explained by a rapid transition to a Wenzel state when the flow is stopped and the memory effect is likely due to the formation of microscopic water pockets as illustrated in **Figure 7.4C-E**. Transition to a Wenzel state is critical at the T-junctions because any slight pinning affects the droplets size and injected volumes. At lower drop velocity of $12\text{--}24\text{ cm min}^{-1}$, pinned droplets are not dislodged but act as nucleation sites for the impregnation of the surface by water, thus disrupting droplet flow.

Figure 7.4 Wetting transition of aqueous droplets in fluorocarbon oil on DTS coated channels. (A) Normal droplets generation under dynamic conditions. (B) After pausing the flow, part of the droplets remains pinned. (C-E) Schematic representation, (A) Under dynamic conditions, viscous entrainment maintains a lubrication layer between the droplet and the wall, (B) When the flow is stopped, the layer vanishes and droplets rapidly transition to the Wenzel wetting regime, (C) Upon resuming the flow, pinned droplets are broken or deform until the channel is only partially obstructed.

Figure 7.5 (A) Scheme to increase PMMA surface roughness by solvent swelling. (B-C) Low and high magnification AFM images of PMMA exposed to 60% acetone for 60 s. (D-E) corresponding images after subsequent SiNPs coating.

As discussed in **Chapter 5**, for a given combination of surface and liquids, the Cassie regime can be made more stable by increasing the surface roughness (**Equation 5.3**), with trade-off of the optical transparency. Increasing the roughness of the SiNPs coating is not possible, however the underlying PMMA substrate can be roughened by solvent processing as illustrated in **Figure 7.5A**. Superhydrophobic surfaces have previously been obtained by swelling polymer surfaces with solvents followed by rapid precipitation [24]. While simple, it renders the surface white and too much swelling would also clog microchannels. Instead, a slight increase in surface roughness was thought to promote a Cassie wetting regime for water in fluorocarbon system (i.e. not necessarily water in air). The same hydrophobic surface modification as previously was then applied. Acetone swelled and

clogged PMMA channels after <30 s. Acetone diluted with ethanol, 3:2 v/v acetone:EtOH allowed the processing time to be varied up to 3 minutes. **Figure 7.5B-C** shows how multiple scales were affected after 60s treatment. The surface was porous with filamentous features at 100 nm scale as well ~150 nm high peaks and valleys on a few μm lateral scale. Ensuing SiNPs coating covered the smallest features while retaining the micron scale topography as shown in **Figure 7.5D-E**. SiNPs are deposited through adsorption on PMMA thus providing a conformal coating that retains topographical features larger than the particle size.

The surface roughness was then optimized to use fluorinated continuous phase. As seen in **Figure 7.6**, surface roughness increases with treatment time but also reduces transparency. Roughness along the channel length was not uniform as evident from scattering of the excitation light. It is difficult to avoid because a solvent concentration gradient is temporarily formed when flushing the channel to precipitate the swollen layer. From a practical perspective, 60 s treatment was the longest time that still allowed tracking of the droplets along the entire channel (see **Chapter 4** for details on image processing). Beyond that too much light was scattered, thus saturating the camera. The small (15 nm) increase in surface roughness was however enough to promote a Cassie wetting regime and flow could be stopped and resumed without immediate pinning. While not the most ideal, this approach allowed testing the toxicity of PCP on bacteria using fluorocarbon as continuous phase. With fluorocarbons, the coating degraded (pinning) over 3-4 hours of usage, in comparison with hexadecane the same chip could be used >72 hours. Drying of the channel did not restore the performances, suggesting removal or fouling of the hydrophobic layer and not adsorbed water pockets. Self-assembled hydrophobic silane monolayers are fairly stable in air (and presumably in oil) but degrades when immersed in water due to hydrolysis [25]. Partial contact between the water droplet and the silane layer could therefore act as nucleation sites for its degradation through hydrolysis.

Figure 7.6 (A) PMMA roughness as function of acetone treatment time. (B) Droplets in fluorocarbon oil in microfluidic chip treated for 60 s prior to hydrophobic modification. (C-D) AFM images of PMMA exposed to 60% acetone for 30 and 120 s.

7.3.3 Van Der Waals Interactions Are Repulsive Across Hydrocarbons but Attractive Across Perfluorocarbons

Van der Waals forces on the water and channel interface were semi-quantitatively compared by their Hamaker constants for different combinations of oils and solid surfaces. According to Lifshitz theory, the Hamaker constant is negative when the dielectric properties of the intervening phase, the oil is intermediate between that of the two interacting media, the solid surface and water droplets [12]. Hamaker constants can be estimated from materials dielectric constant ϵ_0 and refractive index n as per **Equation 7.1** in the absence of dielectric function data. Constants for relevant materials are shown in **Table 7-2**. FC-43 is representative of other perfluorocarbons in **Table 7-1** with similar n and ϵ_0 . Constants for DTS monolayer were approximated as crystal hydrocarbon surface following the approach taken by Geerken et al. [26], which is also comparable to polyethylene (PE). Values for FDTs were approximated by Teflon-AF2400, an amorphous copolymers of

perfluoro(2,2-dimethyl-1,3-dioxole) and tetrafluoroethylene having similar surface free energy as FDTS monolayer [27]. $\nu_e = 3E15 \text{ s}^{-1}$ was used as the maximal adsorption absorption frequency in the UV [12].

Table 7-2 Refractive indices and dielectric constants used for calculation of Hamaker constants.

	Refractive index (n)	Dielectric constant (ϵ_0)
Water	1.333	80
Hexadecane	1.423	2.05
FC-43	1.29	1.9
HFE-7500	1.29	5.8
PTFE	1.359	2.1
FEP	1.344	2
DTS $\approx$ PE $\approx$ hydrocarbon (crystal)	1.5	2.25
FDTS $\approx$ Teflon-AF2400	1.29	1.9

As shown in **Figure 7.7** the estimated Hamaker constant for water-hexadecane-DTS system is negative, indicating repulsion between water and the solid surface. In contrast, Van der Waals interactions are positive for fluorocarbons on DTS and at $\text{Ca}=0$, water is expected to partially or completely wet the solid depending on whether the drop is in Cassie or Wenzel state, respectively. Although contact area can be minimized by increasing the roughness, attachment points for biofouling remain [28]. This fundamental difference is consistent with previous observation that DTS coating is less durable in practice when fluorocarbons are used instead of hexadecane. Although good quality FDTS monolayer could not be prepared inside microchannels, theoretical Hamaker constant indicates that it might not be enough to repulse water droplet in FC-43. Teflon-AF2400 (used to approximate a FDTS monolayer) has one of the lowest refractive index, close to the theoretical limit for solid organic polymers [29]. Therefore, coating leading to repulsive interaction with water across perfluorinated liquids considered in this study might not be physically possible. Note that Teflon-AF itself is not a practical option regardless because it dissolves in fluorocarbons such as FC-43. HFE-7500 results in repulsive interactions on

fluorinated surfaces because of its higher dielectric constant but has other disadvantages as addressed in the next section. This analysis does not consider electrostatic, steric or solvation effects. Additional capillary pressure in closed channels or the presence of solute in aqueous droplets is also not considered. Nevertheless, it provides a relative comparison between combination of liquids and microfluidic surface in order to minimize droplet pinning.

Figure 7.7 (A) Estimated Non-retarded Hamaker constants for water in oil on different substrates. (B) Expected wetting regimes under static conditions.

7.3.4 Perfluorocarbon Oils Significantly Reduces Extraction of PCP from Aqueous Droplets Compared to Hydrofluoroether

The effect of different continuous phases on the apparent toxicity of a model organic toxicant PCP was predicted based on Hansen solubility parameters and investigated experimentally with distribution coefficients and inhibition of resazurin reduction. This pesticide is often encountered in wastewater and has a reported $\text{LogD}_{\text{oct/wat}}$ in range 2-3.3 at pH 7 and a pK_a of 4.8 [30-31]. Distribution coefficients were first measured between PBS and the liquids in **Table 7-1**. As shown in **Figure 7.8**, the molar absorptivity corresponding to the different absorption peaks in predominantly ionized (PBS pH 7.2) and non-ionized (hexadecane) forms respectively are in good agreement with previously reported values [32]. PCP concentrations were estimated from peaks

with absorbance in range 0.1-1 whenever possible; 249 nm peak was used for PBS and 302 nm for hexadecane. Concentration in fluorocarbons was substantially lower but fluorocarbon baseline was too high for the most sensitive peak at 214 nm thus the shoulder at 230 nm was used.

Figure 7.8 Absorbance calibration curves for PCP in (A) PBS and (B) hexadecane.

The distribution coefficients ranked as hexadecane > HFE > FC with no significant difference between the 4 FCs tested as reported in **Figure 7.9**. This trend matches the Hansen solubility distances between PCP and the continuous phases predicted in **Table 7-1** from their respective dispersive (δ_d), polar (δ_p), and hydrogen-bonding (δ_h) components [33]. This model can help in selecting the continuous phase with the lowest solubility (i.e. largest Hansen distance) towards a molecule of interest. It however could not estimate the distribution coefficients because it does not account for ionization in water. Given an approximate oil-water volume ratio of 2 used in our experiments and neglecting non-equilibrium conditions, 94%, 69% and 15% of the original PCP concentration is expected to remain in water droplets when using FCs, HFE and hexadecane as continuous phases respectively. Toxicity would be further underestimated at lower pH where, in non-ionized form ($\text{pH} \ll 4.7$), PCP $\text{LogP}_{\text{oct/wat}}$ increases to 4.7, thus shifting the equilibrium towards the oil phase [30-31]. Nevertheless, neutral pH was chosen in this study as it is more relevant to our assay conditions and the operating condition of anaerobic digesters. Since oils could be classified in 3 categories based on logD, we further compared the apparent toxicity of PCP on *E. faecalis* using one of each: FC-43, HFE-7500 and hexadecane.

Figure 7.9 PCP distribution coefficients between PBS and various oils.

As shown in **Figure 7.10** the apparent PCP EC₅₀ increases from 6.3 mg L⁻¹ in FC-43 to 31.3 mg L⁻¹ in HFE-7500 and was above the highest concentration tested (60 mg L⁻¹) in

hexadecane but could be extrapolated as 277 mg L⁻¹. Although the trend is consistent with measured distribution coefficients, difference in apparent EC50 are 4 to 7 times greater than predicted. Even if an unrealistically high oil to droplet ratio of 10 was considered, difference would still be twice higher than predicted. This discrepancy can be attributed, at least partially to additional solutes from bacteria culture present in the droplets. For instance an increase in ionic strength can shift the equilibrium towards the oil phase [30]. From partition and toxicity experiments with the 6 continuous phases we considered, we can conclude that the use of perfluorocarbons minimizes leaching of PCP from aqueous droplets and other hydrophobic compounds in general. Therefore, the choice of continuous phase between these four can be based on other parameters such as viscosity, gas solubility, etc.

Figure 7.10 Effect of continuous phase on the dose response of *E. faecalis* to PCP assessed by resazurin reduction in microfluidic format.

Figure 7.11 Resazurin reduction by *E. faecalis* spiked with different PCP concentrations, measured in microplate by Dr. Chen Jianlin. Resazurin concentration was 100 μM versus 10 μM used in microfluidic experiments. Inset shows the reduction rate in the first 10 minutes.

The apparent EC₅₀ measured with FC-43 and even HFE-7500 are lower than 77 mg L^{-1} obtained in previous experiment on microplate (i.e. without oil) shown in **Figure 7.11**. As detailed in **Chapter 6** it can be attributed to a higher resazurin concentration used in microplate, 100 μM instead of 10 μM for the microfluidic platform as well enhanced mixing in droplets. In addition, the EC₅₀ = 6.35 mg L^{-1} measured in microfluidic platform under optimized conditions (i.e. using FC-43 and 10 μM resazurin concentration) is comparable to the EC₅₀ = 10 mg L^{-1} towards *E. faecalis* we previously measured based on growth inhibition [34]. This value also compares well with the PCP EC₅₀ = 2 mg L^{-1} to anaerobic sludge that we estimated from methane production inhibition, thus indicating that our microfluidic platform paired with *E. faecalis* as reporting organism is a suitable early warning system for anaerobic bioreactors. Furthermore, testing with both hexadecane and fluorocarbon as continuous phase can help distinguish between inhibition from polar compounds (same apparent toxicity) and non-polar compounds (more toxic in fluorocarbon), thus providing cue on which remediation method to use. For instance, non-

polar compounds can be removed by powdered activated carbon treatments while polar ones are more likely to be heavy metals and ion exchange would be more suitable [35].

7.4 Conclusion

Complete wetting of microfluidic channel by fluorinated continuous phase could not be obtained with fluoroalkylsilane because of aggregates formations but was achieved with alkylsilane coating and fine tuning of the surface roughness to increase droplet mobility while still allowing optical measurements. For stability > 4 hours, complete wetting should be maintained under static conditions. Theoretical analysis of Hamaker constants indicates that this could be achieved through repulsive interactions with a good quality FDTs monolayer and HFE as continuous phase but not the perfluorocarbons considered in this study. A FDTs monolayer would however minimize Van der Waals interactions compared to DTS thus presumably increasing the lifetime of the coating.

We also showed that compared to hexadecane, all fluorinated liquids could reduce partitioning of our model hydrophobic toxicant PCP into the oil phase. Apparent toxicity of PCP to *E. faecalis* using the resazurin assay with FC-43, HFE-7500 and hexadecane were compared and while better than hexadecane, HFE-7500 still underestimate PCP toxicity. FC-43 performed the best and the estimated $EC_{50} = 6.35 \text{ mg L}^{-1}$ means that the dynamic range is well matched with the need for early warning for anaerobic reactor (CH_4 production $EC_{50} = 2 \text{ mg L}^{-1}$). With the transition from hexadecane to perfluorocarbons as continuous phase, the resazurin assay in droplets format allows detection of heavy metals as well as organic toxicants within minutes and provides information on dilution required to prevent inhibition. Using both hexadecane and fluorocarbon can also help in selecting remediation techniques. Future work should focus on improving long term stability towards biofouling, in particular with fluorinated oils, further validation with a broader range of toxicants and tailoring the detection range with various reporting organisms.

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Chapter 8

Summary and Future Suggestions

This chapter summarizes the results presented throughout the thesis and reevaluates the starting hypothesis. Implications and suggestions for improvements are also presented. The resazurin assay was optimized in segmented-flow microfluidic format and toxicants were detected within 3 minutes with better sensitivity than analogous microplate experiments. The resazurin reduction kinetics was attributed to multiple enzymes exhibiting growth phase dependence. Hence sensitivity towards different toxicants is drastically altered by the assay conditions which can be used to tune the response towards specific applications but also advocates for some caution when interpreting results of viability assays in general. Furthermore, the assay in droplet format was enabled by a novel surface modification technique relying on the unique stability of silica sols at their isoelectric point to adsorb a dense silica nanoparticles layer on PMMA. Perfluorocarbons as continuous phase were also demonstrated to minimize the extraction hydrophobic compounds. Results of surface modification and oil-water partition were analyzed with Hamaker constants and Hansen solubility parameters respectively, henceforth providing a theoretical basis for future improvements and more generally, selection of materials for droplet microfluidic.

8.1 Summary

8.1.1 Continuous Droplet-Based Assay for Real-Time Toxicity Monitoring

Combining a cell viability assay and droplet microfluidic for real-time gross toxicity detection and quantification has been proposed in this thesis. The resazurin assay was selected for its potentially fast response, low cost and because it only requires one reagent and one step which simplify its transfer to microfluidic droplets. *Enterococcus faecalis* was selected as a model organism of anaerobic digestion; a potential application that could benefit from early toxicity warnings. Conventional assay protocols are labour intensive and consume too much reagents to be considered for online monitoring. Therefore, a microfluidic platform was design to perform all required functions in ~100 nL droplets, including sample serial dilution, pre-incubation with bacteria followed by addition of resazurin and time course fluorescence measurements. Time course fluorescence intensity curves were obtained with 0.1 s temporal resolution and for 50 to 100 droplets in parallel allowing robust determination of initial reduction rates.

Automation alone was however not enough because conventional protocols are based on incubation time ranging from 30 min to 24 hours. The assay had to be performed under alternative conditions including, short toxicant exposure (< 30 s), high bacterial density (3×10^7 cell mL⁻¹) and moving droplets for continuous mixing. These conditions were not investigated before not only because of technical difficulties in preparing and measuring samples with better than one second accuracy but also because it does not follow recommended procedures, therefore, a detailed validation was required. A linear relationship was confirmed between cell density from 3.7×10^6 to 9.2×10^8 cell mL⁻¹ and resazurin-to resorufin reduction rate. The reduction rate was found to increase because of mixing in moving droplet up to a critical velocity of 4 mm s⁻¹. This effect was attributed to a transition from diffusion limited to enzymatic limited regimes. Alleviating diffusion limitations was shown to result in greater sensitivity to toxicants compared to microplate experiments. For instance, the apparent EC₅₀ of PCP to *Enterococcus faecalis* was 6.3 mg L⁻¹ in optimized droplet format compared to 77 mg L⁻¹ on microplate. A detailed analysis

of the resazurin kinetics as function of concentration was undertaken to provide a rational for its optimal concentration. Michaelis-Menten like behaviour was observed and modelled with the contribution of 2 enzymes having Michaelis constants differing by a factor of more than 20. It was also demonstrated using copper and mercury ions as model hydrophilic toxicants that the choice of resazurin concentration and growth phase alter the relative toxicant sensitivity. From these findings, optimal resazurin concentration is chosen based on the sensitivity required and the apparent Michaelis constants of the reporting microorganism, rather than a simple linearity criterion or fluorescence signal maximization. This flexibility allows it to be tailored to numerous real-time toxicity evaluations, on the assumption that the toxin has an instantaneous (metabolic) action on the living microorganism.

Inhibition of resazurin reduction in optimized droplet format was also compared with proliferation assays. Inhibition of resazurin reduction was comparable with *E. faecalis* growth inhibition for PCP ($EC_{50} = 6.35 \text{ mg L}^{-1}$ and $EC_{50} = 10 \text{ mg L}^{-1}$ respectively) but was more sensitive towards heavy metals particularly copper and zinc. These results advocate for some caution when interpreting resazurin assay data; inhibition of reductase enzymes responsible for resazurin metabolism does not reflect cell cytotoxicity in all cases, as the 'toxin' may antagonize genomic processes or inhibit upstream cascades. The versatility was demonstrated by replacing bacteria with *Saccharomyces cerevisiae* (aka Baker's yeast) as the reporting microorganism. Commercially available yeast has a long storage life, is readily activated in warm water, and requires no unique culture mediums. This low-cost bioreporter makes it an ideal candidate for long-term monitoring with an observed EC_{50} 5 times lower towards Cu^{2+} than *E. Faecalis*. Yeast was less sensitive towards Hg^{2+} and Co^{2+} while addition of millimolar concentrations of Ni^{2+} and Zn^{2+} resulted in higher reduction rate than in control. The latter result could be explained through activation of metal dependent enzymes, reductase upregulation or metabolic response to toxic shock (i.e. efflux proteins). It supports our hypothesis that *toxicants have a unique response to multivariate (microorganism, growth stage, resazurin concentration) analysis of their dose-response relationship* and suggest new approach towards toxicant identification. Identification of pollutants has been investigated with increasingly large

arrays of genetically engineered bioreporters: 5 [1], 15 [2] and even up to 2000 reporters [3], each providing one variable. Our results suggest an alternative approach where one or a limited selection of microorganisms are assayed with respect to more than one variable (e.g. sample and resazurin concentration, growth phase, etc.). For instance, at least 4 characteristic parameters can be extracted from a dose response curve: EC50, Hill slope, maximum, minimum asymptotes and provides the basis for identification of toxicants or classes of toxicant with differing specificities.

Overall these findings support our main hypothesis that *measuring the kinetics of microbial reductases via droplet generating microfluidic chip allows real-time dose-dependent influent toxicity assessment*. Note that measured inhibition does not distinguished between a decrease in metabolic activity or viable cell number and therefore does not directly correlate to cell death. Although the resazurin assay is most often referred to as a viability assay, following the distinction made by Kwolek-Mirek et al. [4], it would be best classified as a vitality assay. This is not a problem in the context of water testing were the fate of the reporting microorganisms is not paramount but may limits other potential applications such as antibiotic testing. The model toxicants were chosen for the sake of literature comparisons and although unique sensitivity patterns were observed between Baker's yeast and *E. faecalis*, the microfluidic based assay is not specific to these toxicants and bioreporters. Unknown toxicants and microorganism milieus can be readily assessed within the given parameters to toxicants and bioreporters; 1) Toxicants remain soluble and do not partition into the continuous oil phase, 2) Inhibition from unknown toxicants or mixture will be reported as function of the dilution factor instead of absolute concentration, 3) Bioreporters display reduction kinetics >10x than background or solvent mediated reduction, 4) Bioreporters can homogenously be dispersed with no immediate aggregation.

8.1.2 Selection of Continuous Phase and Surface Properties for Segmented-Flow Microfluidic

Surfactants added to the oil phase are the primary method of stabilizing droplets in segmented-flow microfluidic. A major disadvantage of surfactant stabilization is their

tendency for cross contamination between droplets and complicated droplet fusion. After many failed efforts to employ common hydrophobic surface modifications, a novel technique was developed for PMMA (and other thermoplastics) that eliminated the need for surfactant while sustaining continuous droplet-production/reagent addition involving bacterial and yeast suspensions [5]. The novel approach relied on the stability of silica sols at their isoelectric point to adsorb a dense silica nanoparticles layer on the post-processed microfluidic chip. Silica surface coverage was shown to be correlated with the number of coating cycles and inversely correlated with the nanoparticles size. This layer made the channel hydrophilic and oil in water (o/w) droplets were generated. This silica acts as a primer layer for subsequent flexible surface modification. In this regard, Dodecyltrichlorosilane (DTS) or 1H,1H,2H,2H-perfluorodecyltrichlorosilane (FDTS) modification successfully prevented aqueous droplet pinning in hexadecane under both, static and dynamic conditions. The partitioning of hydrophobic compounds in hexadecane drove our search for fluorocarbon surface treatments and oil phases to expand the assay beyond hydrophilic toxicants. However, FDTS surface modification onto silica did not prevent pinning in fluorinated liquids and it was attributed to the aggregates in the FDTS monolayer based on AFM analysis. As an alternative, aqueous droplets in fluorocarbons could be generated with DTS coating and surface roughness increased from 8 to 24 nm and allowed successful microfluidic operation for up to three hours. Our hypothesis that *hydrophobic surface modification of the channel walls prevents wetting of the walls by the dispersed aqueous phase without surfactant additives* is validated for hexadecane but only under limited timeframes for fluorocarbon oil phases. Theoretical analysis of Hamaker constants suggested the presence of repulsive Van der Waals interaction for water-hexadecane-DTS system but that even with a perfect FDTS layer, forces will remain attractive for the perfluorocarbons used in this study. Repulsive forces are important to thermodynamically prevent wetting of the walls which minimizes the risk of fouling in the long term. Fortunately, analysis of Hamaker constants suggest that this condition can be fulfilled by other fluorocarbons as elaborated in the next section.

Limitation introduced by partitioning in the continuous oil phase were also addressed. It is well known that fluorinated liquids can minimize extraction of non-fluorinated compounds

from droplets. Previously reported literature and commercial sources for droplet microfluidic however seemed to indicate that perfluorocarbons (FC) and hydrofluoroether (HFE) can be used interchangeably. On the contrary, Hansen solubility distance of our model toxicant PCP ($\text{LogD}_{\text{oct/wat}}$ 2-3.3 at pH 7) predicted the solubility of PCP in HFE to be intermediate of that in hexadecane and FC. Direct impact on distribution coefficients was confirmed by analytical measurements and indirect artefactual contribution to the measured toxicity in droplet assay was quantified as PCP $\text{EC}_{50} = 6.3 \text{ mg L}^{-1}$ in FC-43 versus 31.3 mg L^{-1} in HFE-7500 and 277 mg L^{-1} in hexadecane which validates our hypothesis that *larger Hansen solubility distance between the continuous oil phase and toxicant reduces its partitioning from the dispersed aqueous phase and decreases its apparent half inhibitory concentration*. Criteria commonly pushed forward for the selection of HFEs over FCs is their lower global warming potential. Although important, these concerns arose from large amount used in thermal cooling applications and our results advocate for the use of FCs in microfluidic applications whenever non-fluorinated hydrophobic compounds are involved. In relation to biological treatment, testing with both hexadecane and fluorocarbon as continuous phase can help distinguished between inhibition from polar compounds (same apparent toxicity) and non-polar compounds (more toxic in fluorocarbon), thus providing cue on which remediation method to use (e.g. ion exchange, activated carbon, etc.).

8.2 Implication and future suggestions

8.2.1 Long Term Operations with Perfluorinated Continuous Phase

The first step towards long term operations with fluorocarbons as continuous phase is to replace the DTS coating with fluorinated chains but without polymerized aggregates. Fluorinated triethoxysilane or trimethoxysilane are less reactive than their trichlorosilane counterpart and therefore less prone self-polymerization but also form less densely packed monolayers [6]. Alternatively, vapour phase deposition inside microchannel might be improved with shorter chains (perfluorooctyltrichlorosilane) and better control over pressure, temperature and moisture content.

Fluorinated monolayer instead of DTS is expected to reduce, but not reverse interaction between the dispersed phase and the channel surface with FC-43 as continuous phase (**Figure 7.7**). Fluorinated liquids with higher molecular weight like FC-70 (Mw 820) are predicted to have slightly negative Hamaker constants ($-0.01 \cdot 10^{-20}$ J). It might however limit the length of incubation channel because of increased backpressure due to its higher viscosity (FC-70 24 cP vs FC-40 4 cP). Fluorinated siloxanes (e.g. perfluorononyl dimethicone) are another class of liquids potentially useful as far as wetting is concerns, albeit low solubility of hydrophobic molecules should be verified first. If a negative Hamaker constant cannot be obtained and wetting has to be controlled through surface roughness, the following points should be considered.

- While challenging from a fabrication standpoint, for best durability, surface roughness of the bulk material should be controlled rather than using additional layers.
- Materials with the highest intrinsic contact angle (water in oil), yet transparent and insoluble in the oil, are the best candidates since additional chemical surface modification might not be necessary (**Equation 5.3**).
- Refractive index of the chip material closely matching the one of the continuous phase will reduce scattering thus allowing greater surface roughness without impeding optical measurements.

Finally, if absolutely necessary fluorosurfactants can be added in concentration that do not prevent droplet coalescence.

8.2.2 Anaerobic Sludge as Reporting Organisms

E. faecalis is relevant to wastewater treatments because it is abundant in anaerobic digestion but only represents part of the digestion process [7]. As an alternative, Chen et al. previously demonstrated that, on microplate, the resazurin assay can monitor anaerobic sludge [8]. Replacing bacteria by sludge was attempted in droplet format but was complicated by sludge flocculation. The sludge was vigorously shaken to break the aggregates and was left to settle for 10 minutes before sampling to prevent injection of

remaining millimeter sized aggregates that would clog microchannel. Despite the presence of intermediate size particles as evident from scattering, resazurin reduction was not observed within 5 minutes, even without pre-dilution, as shown in **Figure 8.1**. The density of active organisms was considerably reduced compared to microplate incubation because large aggregates had to be removed. Oxygen contamination during transfer is also possible, however resazurin reduction by facultative anaerobic sub-populations would still be expected.

Figure 8.1 Resazurin reduction test with anaerobic sludge.

Preprocessing the sludge to break aggregates is necessary, but any de-aggregation methods are likely to affect cytotoxicity, interfering with the primary goal of the assay. Mixture of organisms are potential indicator of whole digestion process, however the cascade of events leading to inhibition of the entire microbial population takes time (e.g. acetic accumulation). Hence at short timescale toxicity affecting the most sensitive organisms can be masked by resazurin reduction by the rest of the population. In that regard, methanogenic archaea would be a good indicator as it is often considered as the most sensitive species in anaerobic digestion [9]. Their inherent sensitivity requires demanding culture conditions that make it impractical for routine sensing towards industrial applications. As an alternative, activity of enzymes (e.g. coenzyme M, methanofuran) only found in archaea could be probed by

monitoring specific substrate formation/disappearance instead of resazurin [10]. In-situ measurements might however be limited by availability of fluorescent substrates.

8.2.3 Increasing the Sensitivity

As discussed in this thesis, *E. faecalis* under our assay conditions is a good reporter for anaerobic digestion as it matches well with inhibition of methane production. Other potential applications such as effluent discharge or drinking water monitoring require better sensitivity. There are several ways it could be improved:

- Longer toxicant exposure. The length of the pre-incubation channel could be changed to increase the time organism are exposed to toxicant before adding resazurin. Although there is not much room to play, an increase from 20 s to 2-3 minutes could make a difference, especially under continuous mixing.
- More sensitive organisms. Other type of bacteria or eukaryotic cells can be used. In particular, microalgae or protozoa could be more sensitive than yeast towards metals other than copper [11]. Mammalian cells can also be assayed with resazurin and would be more relevant to human toxicity. Cells that can be grown in suspension would be the best candidates.
- Operating at kinetic limit. Growing cells are not convenient to maintain in the long run, instead different carbon sources could be added to stationary phase at the beginning of the assay. As pointed out in **Chapter 6**, caution is however advised when multiple enzymes are involved.
- Increased membrane permeability. Toxicant concentration inside the cell can be substantially lower than in bulk. For sensing purposes, additives altering cell permeability might increase the bioavailable fraction.

8.2.4 Beyond water toxicity

Our microfluidic platform could also be used for rapid drug screening. Screening on whole cell would however require a viability indicator that better correlates with cell deaths, possibly esterase or protease activity. A better understanding of enzymes involved in

resazurin reduction as well as inhibition mechanism would also be helpful. Resazurin reduction can be studied in vitro with pure enzymes or subcellular fraction [12]. The location of these enzymes in the cell can be determined by confocal microscopy and selective inhibitor (e.g. Cu^{2+} , Hg^{2+}) which would help in interpreting inhibition data.

Combining water, hydrocarbon and fluorocarbon creates triple phase systems which might be useful to study biphasic reactions as shown in **Figure 8.2**. For instance, small hexadecane droplets in contact with water droplets could be used for continuous delivery of poorly water soluble drugs or substrates such as fluorescein-diacetate.

Figure 8.2 Water, hexadecane, fluorocarbon triple phase systems in channels modified with silica and, (A) DTS (B) FDTS.

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